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VIABILITY OF SKIN IN RELATION TO VARIOUS METHODS OF STORAGE*

M. Allgöwer† and T. G. Blocker, Jr.

The possibility of storing human skin in a viable state is of increasing clinical interest. In order to reduce hazards to the patient of repeated operations, it is often desirable to take at one sitting the skin required for successive autografts to cover burned surfaces. Also, homografting as a life saving procedure in extensive burns constitutes another use for stored skin and skin banks would serve a most useful purpose in the event of a large burn disaster.

For many years there have been reports on skin storage. As early as 1881, Girdner tried to use stored skin for homografts, and Brewer (1882) succeeded in doing so. His skin was stored for 36 hours before being used. More detailed work was done by Wentscher from 1896 to 1903. He first made transplants of homologous and autologous skin, stored for two days in saline. Later he published microscopical analyses on skin stored below 10° C. and returned to the same patient after 7–14 days' storage in wet gauze in test tubes. Ljunggren (1898) published results obtained in 22 clinical cases of transplants of preserved skin. He had good initial takes with homologous tissue which had been stored in ascitic fluid up to four weeks. Microscopical studies of biopsies revealed mitotic activity. Enderlen (1898) repeated Wentscher's experiments but obtained survival of only four days when he stored skin in wet gauze. Dried parts of the skin proved unsuccessful in transplantation. Carrel (1912) stored dog skin as well as human fetal skin in petrolatum at ice box temperature. Dog skin stored for one to ten days took well. Homografts with homologous fetal skin from

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†Fellow of Swiss Academy of Science; home address: Bürgerspital Basel, Chirurgische Universitätsklinik, Basel, Switzerland.

man, stored as long as 14 days, were said to take as well as fresh ones, and even after seven weeks' storage there was still some take. Kubanyi (1930) had one positive transplant of autologous skin stored in the patient's own serum and no take with vaseline-stored skin. Filatov (1931) used refrigerated cadaver skin after several days of storage but the transplants were not permanently successful.

More recently, the use of stored skin as a clinical routine has been demonstrated by Webster and Mathews. Webster in 1944 published results obtained upon 23 patients with 36 grafts of skin which was stored partially backed with Pliofilm and folded together so that the raw surfaces were in contact. The skin was then wrapped in Pliofilm and this subsequently wrapped in xeroform or vaseline gauze. This skin took in transplants very well after having been stored up to two weeks, but did not take after more than three weeks' storage. Mathews (1945) advocated a somewhat similar storage method in vaseline gauze. On granulating surfaces he had takes up to three weeks, and on clean operative surfaces, in one case, up to two months. Tissue culture studies eight days after storage yielded outgrowth of a few fibroblasts. Fluid media such as Ringer's solution, liquid vaseline or paraffin were not found suitable by these authors.

There have been attempts to work with skin stored at low temperatures. Mider and Morton (1939) found that rat epithelium and connective tissue cells may still survive, in part at least, when frozen to —74° C. Briggs and Jund (1944) had 52% take of grafts with mouse skin frozen within 1.8 to 2.2 minutes from 0° to —15° C. and kept at —78.5° C. for one to 24 hours. Controls with normal skin yielded 100% positive takes. Strumia and Hodge (1945) froze small pieces of human skin in citrated plasma during 15 to 30 minutes down to —20° to —25°C. The skin was kept this way up to 61 days. On three patients Strumia got 80.5% take after placing the stored skin on clean granulating surfaces. Webster in one case used skin refrigerated to —72° C. and kept at this temperature for 17 days, whereas another piece was lyophilized after freezing. Only the latter took and then it was less satisfactory than the

control fresh skin. There has been considerable work done on the survival following different methods of slow and rapid freezing on malignant cells to temperatures as low as —253° C. There is no doubt that in many instances some of the cells remain in a viable state, but it is generally agreed that there is a very marked loss of vitality in the frozen tissue as revealed by diminished infectivity of tumor cells or reduction in outgrowth in tissue culture of both normal and tumor cells.

The vaseline or wet gauze storage of skin introduced by Carrel and worked out as clinical routine by Webster in America and by Mathews in England has become the usual storage method for skin in recent years. This method, having been shown to be of good practical value, however, has not been proven to be the best way of storing skin. Hanks and Wallace (1949), working with rabbit skin stored in mineral oil on one hand and in 10% serum on the other, found that the survival in the latter was better at both 0° and 8° C., 6–8° C. giving the best results. Maragoni (1950), comparing results obtained with tissue culture on rabbit skin stored in 10% serum, piloform *(sic.)* and at low temperatures, found survival time longest with the use of 10% serum. Grafting experiments with rabbit skin apparently gave similar results. No comparable studies have been published on human skin previous to the present paper. For this reason it was thought of interest to investigate the following questions: How does the usual storage method in vaseline or wet gauze at ice box temperature compare with storage in 10% serum or at a low temperature? The approach was sought in studies on tissue cultures of skin and in comparison of the tissue culture results with the actual take *in vivo*. The superiority of the 10% serum storage soon became evident (Tables I and II), which led to two other questions:

How does the viability of skin compare in storage with vaseline gauze or with wet gauze?

Can 10% serum in balanced salt solution be improved upon by other fluid media?

Materials and Methods

Tissue Culture Techniques

Human skin

Split-thickness skin obtained from the operating room was put within a few hours into hanging drop cultures according to the method described by Lewis, Pomerat and Ezell (1949). The plasma clot consisted of one drop of rooster plasma containing 6000 units of penicillin per ml and one drop of diluted embryonic juice, with a fluid phase of four drops of normal human serum. Thus the final concentration of penicillin was 1000 units/ml.

Rabbit skin

After shaving, the skin was cleansed by soap and ether washings. Split-thickness skin was taken either by free-hand knife or by a Padgett dermatome. Pieces about 1.5 mm square were placed in a solid medium of one drop of rooster plasma and one drop of diluted embryonic juice, and provided with a fluid phase of four drops of rabbit serum after the coagulation of the clot. Penicillin at a final concentration of 1000 units/ml was employed. As rabbit skin, unlike human skin, gives fibroblasts as well as epithelial cells when cultivated in hanging drops, both kinds of cells have been evaluated.

Storage Methods

Human skin

Vaseline gauze: Split-thickness skin was cut into pieces 1 cm square and folded together so that the raw surfaces were in contact. This skin was wrapped tightly in vaseline gauze, put into several Kahn tubes and kept at 5° C., to be tested at different times, one skin sample being used only once. The same procedure was used for the skin stored in the deep freeze (—37° C.).

Wet gauze: The same procedure as for vaseline gauze was used, the folded skin being wrapped in gauze wet with balanced salt solution (Gey's) so that the skin was not actually immersed

in fluid but was kept in a moist atmosphere in centrifuge tubes stoppered to make them airtight.

Serum 10%: Ten pieces of skin each 1 cm square were placed in 25 ml of storage fluid containing 10% serum and 90% Gey's, with 1000 units of penicillin/ml and phenol red at a concentration of 1:200,000. This skin was stored at 5° C. in 50 ml flasks and the storage fluid changed each week. In other experiments using serum, the amount of the storage fluid was varied, as well as the serum concentration and the frequency of changing the storage fluid. These conditions will be described in the corresponding experiments.

Rabbit skin

The abdomens of four rabbits were shaved, the skin washed with soap and ether, and a half drum of skin (Padgett dermatome) of split-thickness was removed. Pieces of equal thickness, measuring about 2 cm square were cut out and stored under two conditions at 5° C.:

Vaseline gauze: the skin was prevented from immediate contact with the vaseline by a thin layer of wet gauze and then wrapped tightly in vaseline gauze as described for the human skin.

Rabbit serum 10%: Two pieces of skin 2 cm square were put into 15 ml of the following storage fluid: 10% rabbit serum and 90% balanced salt solution (Gey's) with 1000 units of penicillin/ml and a final concentration of phenol red of 1:200,000. After 14 days' storage, both kinds of stored skin were returned to the back of the animal after full-thickness excision of a corresponding area. The skin was sewn in and deep sutures were placed to exert slight compression on the graft. The dressing was sewn on and remained in place unchanged for five days.

A Recording of Tissue Culture Results

A scale from ± to 4+ was used to record the outgrowth of the cultures, whereby ± referred to slight growth of small sheets or groups of a few cells. Outgrowth which extended across one-fourth of the low power field was called 1+; up to

one-half 2+, etc., up to 4+, when the entire field was covered. The actual magnification was approximately 100x, so that the low power field corresponded to about 1.5 mm.

There were always ten cultures for each experimental condition and the recordings in the different tables were made by totaling the plus readings of the ten cultures. Table III, however, gives one representative experiment with the readings of each culture. Approximately 3000 epithelial cultures were made during this investigation.

Results

Storage in 10% Serum as Compared with Storage in Vaseline Gauze

Tissue culture experiments with human skin

For the experiment with the six skins shown in Table I, it was thought advisable to utilize serum prepared with fresh uncitrated blood. This table shows the results obtained with six different samples of skin that showed positive outgrowth in initial cultures. This outgrowth, while positive in all cases, was of different degrees. Skin stored for 14 days showed outgrowth of about half as much as that shown in the initial cultures of fresh skin; skin stored for 21 days showed outgrowth of about one-fourth as much, and skin stored for as much as 28 days showed some outgrowth in three out of six samples. These results are definitely better than those obtained when the skin was stored in plasma prepared from citrated blood. No skin stored in vaseline gauze at either ice box temperature or at —37° C. showed any outgrowth after 14 days' storage. Figure 1 shows an epithelial sheet (skin no. 2, Table I) grown out of a skin explant which had been stored for 21 days in 10% serum before explantation. The culture was photographed in the living state with phase contrast optics after six days of incubation.

Of special interest is the contamination which occurs under certain conditions in spite of the addition of 1000 units of penicillin per ml to the culture medium. This is especially frequent for skin of patients who have been treated previously

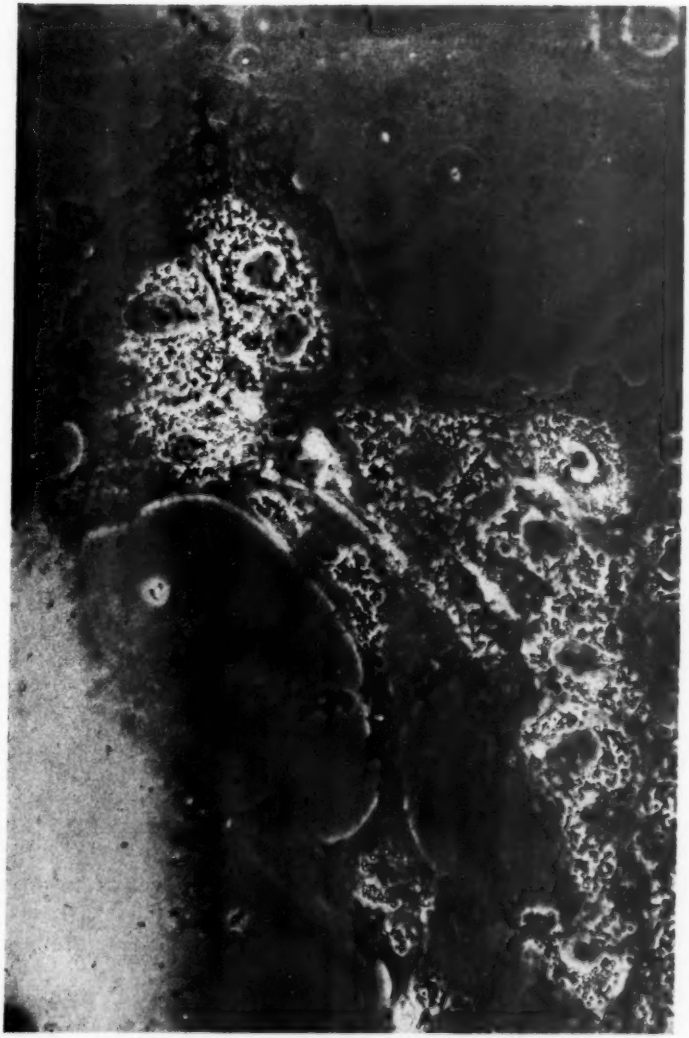

FIG. 1. Portion of living epithelial sheet from a 6 day culture of skin stored for 3 weeks in 10% serum. Phase contrast –500 x).

TABLE I

Effect of various skin storage methods on epidermal outgrowth in tissue culture

Skin source	Skin from	Initial Outgrowth	Outgrowth after storage for 14 days			21 days			28 days		
			VG	S	DF	VG	S	DF	VG	S	DF
1. 22 yr. ♀ Negro	Left arm	16	0	12	0	..	3	..	..	0	..
2. 85 yr. ♀ Negro	Abdomen	16	0	16	0	..	7	..	..	1	..
3. 45 yr. ♀ Negro	Abdomen	27	0	6	0	..	0	..	..	0	..
4. 45 yr. ♂ White	Abdomen	23	0	9	0	..	6	..	..	2	..
5. 35 yr. ♂ White	Abdomen	11	0	1	0	..	0	..	..	0	..
6. 36 yr. ♂ White	Thigh	9	0	6	0	..	7	..	..	2	..
TOTAL OUTGROWTH		102	0	50	0	..	23	..	..	5	..

VG—Vaseline gauze. S—Serum. DF—Deep freeze.

during a long period with penicillin. In such cases the contamination no longer occurred in tissue culture after about two weeks of storage in 10% serum containing 1000 units of penicillin per ml, whereas the vaseline-stored skin still showed very marked evidence of contamination.

Rabbit skin. Comparison between tissue culture results and actual grafting results, after 14 days' storage in 10% serum and vaseline gauze.

Rabbit skin taken with the Padgett dermatome and varying from 0.2–0.5 mm thick was tested for initial outgrowth immediately after being removed. The initial outgrowth of the skin from these rabbits was rather poor as compared to that obtained from other rabbits although the animals were apparently in good condition. In every skin, however, there was formation of epithelial sheets and of fibroblasts. Table II shows that after 14 days' storage in 10% serum the outgrowth was about the same as that obtained in the initial tests on this skin. No epithelial outgrowth occurred following vaseline storage and only a few fibroblasts migrated from the explants. When returned to the animal, the graft of the same serum-stored skin twice gave a complete take and in two instances showed a slight desquamation of the superficial layer of the graft. The vaseline-stored skin sloughed off totally in two cases and gave partial take of the deep layers in the two other samples.

Human skin. Clinical trials.

This skin was prepared according to Mathews' method (wrapped tightly in vaseline gauze and put into glass tubes securely closed). Satisfactory results were generally obtained with skin stored from six to ten days. Patient no. 5 in Table I, who had multiple granulating areas after extensive burns, needed repeated grafts. Pieces of this patient's skin which had been stored 21 days in vaseline gauze, serum and in a deep freeze at —37° C. were placed on granulating areas of the patient. The serum-stored skin gave good initial take but subsequently it partially sloughed off. Vaseline-stored skin showed very poor take and skin stored in the deep freeze showed no take.

TABLE II

Comparison of the viability of stored skin by tissue culture technique and by autografts

Animal	Type of skin	Initial Outgrowth E	Initial Outgrowth F	Outgrowth in tissue culture after 14 day's storage in 10% serum E	10% serum F	VG E	VG F	Results obtained with autografts after 14 days' storage in 10% serum	VG
Rabbit 15	Thin	3.5	5	4.5	7	0	0	Complete take of graft	Graft did not take
Rabbit 16	Medium	1	4	3.5	7	0	0.5	Complete take of graft	Graft did not take
Rabbit 17	Thick	5	6.5	11	6	0	2.5	Desquamation of superficial layer only; graft persisted	Partial take
Rabbit 18	Thick	6	6	1.5	4	0	0	Desquamation of superficial layer only; graft persisted	Partial take
TOTAL OUTGROWTH		15.5	21.5	20.5	24	0	3	Good	Poor

VG—Vaseline gauze. E—Epithelial outgrowth. F—Fibroblasts.

Short Term Viability Studies of Skin Stored in Wet Gauze or in Vaseline Gauze.

The previous results indicated that no viability could be detected by means of tissue culture tests when skin was stored in vaseline or in wet gauze for over 14 days or more. Therefore, the toxicity of vaseline gauze as such was investigated and short term experiments were made to obtain more detailed information regarding the decreased viability of such skin. Table III shows the results of an experiment in which viability was tested every fourth day for 12 days. After four days of storage the lag period and the final outgrowth still were as good as in the initial controls. After eight days' storage the lag period was more than two days in vaseline-stored skin whereas the wet gauze-stored skin showed a somewhat shorter lag period, although the final outgrowth of the skin following both storage conditions was approximately the same but markedly reduced as compared to the controls. After 12 days' storage under either condition, only a few cells migrated from the explants and this occurred seven to eight days after explantation. It seemed of interest to investigate the possible toxic action of vaseline. For this purpose fresh pieces of skin were smeared with vaseline and explanted at the same time as the controls. They generally showed the same amount of outgrowth as the controls and the few cultures that were smaller seemed to be inhibited merely by the mechanical interference of small accumulations of vaseline.

Serum Storage under Various Conditions at 5° C.

Experiments on the following points were made: amount of storage fluid in relation to the amount of tissue, optimal concentration of serum in the storage fluid, and effect of changing the storage fluid twice a week, once a week, and not at all.

Table IV A shows that there is an important relationship between the amount of stored skin and the amount of storage fluid. Too much or too little tissue in a given amount of serum decreased its viability considerably. Experiments in which the amount of tissue was kept constant and the amount of

TABLE III

Short term viability studies of skin stored in wet gauze and in vaseline gauze.

Skin No.	CONTROLS		VASELINE CONTROLS		SKIN STORED IN WET GAUZE FOR						SKIN STORED IN VASELINE GAUZE					
					4 DAYS		8 DAYS		12 DAYS		4 DAYS		8 DAYS		12 DAYS	
					Outgrowth after:						Outgrowth after:					
	2 days	6 days	2 days	6 days	2 days	6 days	2 days	6 days	2 days	6 days	2 days	6 days	2 days	6 days	2 days	6 days
1.	+	+++	+	+	+	+++	—	+	—	—	+	+++	—	+	—	—
2.	±	+++	+	++	+	+++	±	+	—	—	+	+++	—	+	—	—
3.	++	+++	±	++	+	+++	—	+	—	—	+	+++	—	±	—	—
4.	+	+++	±	+	+	+++	+	+	—	—	+	+++	—	+	—	—
5.	+	+++	—	+++	+	+++	+	+	—	—	+	+++	—	+	—	—
6.	+	+++	++	+++	+	+++	+	++	—	—	+	+++	—	±	—	—
7.	+	+++	++	+++	+	+++	—	±	—	—	+	+++	—	+	—	—
8.	+	+++	+	+++	+	+++	—	±	—	—	+	+++	—	±	—	—
9.	+	+++	++	+++	+	+++	—	—	—	—	+	+++	—	++	—	—
10.	+	+++	+	+++	+	+++	±	±	—	—	+	++	—	—	—	—
		30		24		30		8.5		0*		29		8.5		0*

*A few cells migrated after 8 days of culture.

TABLE IV A

Effect of skin-fluid ratio on epidermal outgrowth "in vitro" of stored skin.

Skin stored in 8 ml of 10% serum			
Amount skin in sq cm	Ratio of skin to fluid	Epidermal outgrowth after 7 days' storage	Epidermal outgrowth after 14 days' storage
Maximum (skin barely covered with serum)	Greater than 1	4	0
8	1	12	19
4	0.5	20	19
2	0.25	13	7
1	0.125	9	5
0.5	0.0625	9	2

storage fluid was varied showed the same deleterious effect of too much or too little storage fluid. The ratio of 1 to 2 ml of storage fluid per 1 cm^2 of stored skin seemed optimal provided that the skin was approximately 0.2 mm in thickness.

Table IV B gives evidence that 100% serum is a poor storage medium. Skin stored in 33% serum as well as in 10% serum

TABLE IV B

Effect of serum concentration on epidermal outgrowth of stored skin as revealed by tissue culture.

Four sq cm pieces of skin, each stored in 8 ml of serum		
Serum concentration	Epidermal outgrowth after 7 days' storage	Epidermal outgrowth after 14 days' storage
100%	9	6
66%	15	14
33%	19	21
10%	18	16

gave about the same outgrowth after two weeks' storage. While skin stored in 66% serum always showed about the same outgrowth, the cells appeared somewhat more granular which indicated that this concentration was too high.

Table IV C demonstrates that changing the 10% serum storage fluid markedly reduced outgrowth activity of skin after two weeks storage.

TABLE IV C

Effect of changing storage fluid on epidermal outgrowth "in vitro" of stored skin.

Four sq cm pieces of skin, each stored in 8 ml of 10% serum	
Storage fluid changed	Epidermal outgrowth after 14 days' storage
Twice weekly	7
Once weekly	8
Not changed	17

DISCUSSION

The usual storage method for human skin in recent years has involved the use of wet gauze or of vaseline gauze at ice box temperature. The clinical usefulness of these procedures has been demonstrated by Webster, Mathews and others. Tissue culture studies with human skin as reported in this paper show that these storage methods are far from being optimal. With storage in 10% serum, however, it is possible to obtain positive outgrowth of epithelial cells in tissue culture after 28 days of storage. Using the same method with skin stored in vaseline or wet gauze, demonstrable viability is lost after 12 days of storage.

Animal experiments as well as clinical trials on patients indicate that the results obtained with stored skin in tissue culture and the actual take of grafts *in vivo* with similarly stored skin are directly comparable. Rabbit skin stored for 14 days in vaseline gauze but protected from direct contact with vaseline by a thin layer of wet gauze showed no epithelial outgrowth and only a few fibroblasts in tissue cultures. The take of this skin *in vivo* was very poor. The same skin, stored in 10% serum, gave approximately the same outgrowth in tissue culture after 14 days' storage as it did in initial experiments, and the takes on the animals were excellent. Using human skin, similar results were observed in tissue cultures and on human patients.

Viability was less well demonstrated *in vitro* since the tissue culture test is somewhat more stringent than the test by reimplantation, as evidenced by the fact that *in vivo* tissue may take for about eight days after it has ceased to show outgrowth *in vitro*. This indicates that tissue culture studies are very exacting tests for survival studies of stored skin.

Serum storage for skin proved to be superior to vaseline gauze storage, but certain conditions of serum storage must be fulfillled to make it optimal. The ratio of the amount of storage fluid to the area of tissue is of great importance. Too much as well as too little storage fluid is deleterious to the tissue. Skin which was barely covered with serum showed decreased activity after eight days of storage and so very much resembled the vaseline stored skin. It may be assumed that there is too great an accumulation of catabolites to permit survival for any length of time. Viability of skin in storage appears to be optimal when the square centimeters, using skin approximately 0.2 mm thick, about corresponds to the number of milliliters of storage fluid. This ratio should be easy to apply in routine storage procedure.

The concentration of serum in the storage fluid should not exceed 33% and as 10% serum gives approximately the same result, this amount has the advantage of economy. The harmful effect in storage of 100% serum may be explained by the fact that serum which is known to be a good promoter of epithelial outgrowth in tissue culture may provoke, even at 5° C., too intensive metabolic activity. At a serum concentration of 10 to 33% the stimulating activity on skin is reduced and, therefore, probably accounts for its value in skin storage. In related experiments we have found that the outgrowth promoting activity on skin in tissue culture of 33% serum is about three-fifths that of 100% serum and 16% serum one-fourth that of 100% serum.

The changing of the storage fluid containing serum causes a very marked decrease in the outgrowth activity of the skin after 14 days' storage. This has to be kept in mind when judging the survival times given in this report for they are based on skin with storage fluid changed weekly in an attempt to free

it from catabolites which had presumably accumulated. This assumption proved to be wrong. Changing the storage fluid has the same effect as too large an amount of storage fluid. The factors involved remain unexplained. It is evident that serum storage without the changing of the storage fluid would have proven the superiority of serum over wet or vaseline gauze storage even more conclusively.

There is a growing impression among workers maintaining tissue cultures under conditions of continuous cultivation that a too energetic replacement of fluid nutrient in roller tubes and Carrel flasks, together with washing of the medium with warmed balanced salt solution, has a deleterious effect upon the viability of cell strains. Some workers are employing the technique of removing half of the nutrient fluid after three days of incubation and replacing this with an equal volume of fresh nutrient mixture. This procedure also eliminates culture washing with balanced salt solution. It is argued that at the present stage of knowledge there is no "perfect" fluid nutrient medium but that cells "condition" whatever nutrient is brought in contact with them. The reasonableness of these conclusions is supported by the success of Earle's technique for the cultivation of a clone of cells from an individual element with the use of "conditioned" medium derived from Carrel flask cultures in the phase of their most active proliferation under conditions in which the isolated cell is placed in a capillary tube where diffusion of excreted cellular materials is limited.

While skin stored in 10% serum changes the pH at a regular rate even under storage conditions of 5° C., the desideratum of the replenishment of the storage fluid may be outweighed by the factor that the stored skin may lose its capacity to "condition" the medium repetitively under the particular conditions of storage.

Further studies designed to improve storage conditions at 5° C. should take into account, not only the metabolic turnover, but also the factor of probable contributions from the stored skin to the constituents of the medium. This might be tested experimentally by transferring the skin to a smaller volume of renewal fluid where the factor of "conditioning" would create

minimal demands upon the cells. Another possibility would involve the replacement of only one-half of the storage fluid thus imitating the procedure in vogue for the management of tissue cultures in roller tubes or Carrel flasks. The entire question of medium "conditioning" begs biochemical analysis.

The accessibility of oxygen to the tissue stored at temperatures between 6–8° C. has been shown to be of great importance by Hanks and Wallace. Care was taken, therefore, to employ storage flasks which gave a great surface and a depth of between 10–20 mm to the storage fluid.

Freezing the skin in an ordinary deep-freeze ice box at a temperature down to —36° C. yielded no epithelial or fibroblastic outgrowth in six skin samples after 14 days' storage, although these skins grew fairly well when fresh. The same skin samples had shown positive outgrowth after storage with 10% serum for the same length of time. This does not mean that there is no possible survival of human skin after deep-freezing. Animal experiments with malignant and nonmalignant cells by Breedis, Briggs and others have proven the possibility of survival of these cells down to —253° C. but generally the loss of viability is considerable. In 34 storage experiments with blood vessels by slow and rapid freezing down to —76° C., Pierce *et al.* found positive outgrowth in tissue culture in only two instances. Their grafts with the same deep freeze material yielded very poor results. Considering these facts, as well as our own results, deep freeze storage compares unfavorably with either vaseline gauze, wet gauze or 10% serum as a storage condition where maximal survival is the aim. However, we have made no effort to study the complex physicochemical conditions which are involved in subjecting protoplasm to rapid lowering of temperature.

Summary

Split-thickness human skin stored in 10% serum at 5° C. shows marked superiority in viability as tested with tissue culture technique over that stored in vaseline gauze at 5° C., vaseline gauze at —36° C., and wet gauze at 5° C. Epithelial outgrowth *in vitro* occurs up to 28 days when the skin is stored

in 10% serum. Optimal conditions for serum storage of skin are that the serum concentration be within the range of 10 to 33%, that the amount of storage fluid in milliliters roughly corresponds to the area of the stored skin in square centimeters, and that the fluid not be changed during the storage period. Comparison between results with tissue cultures and those obtained with regrafting the skin back to patients or to experimental animals confirms the value of tissue culture tests. The results of both methods are comparable, but tissue cultures prove to be somewhat more stringent indicators of the viability of stored skin and are accompanied by fewer variable factors.

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BIBLIOGRAPHY

Breedis, C.: The action of extreme cold on leukemic cells of mice. *J. Exp. Med.*, 76:221, 1942.

Breedis, C., and Furth, J.: The possibility of preserving neoplastic cells in the frozen state. *Science*, 88:537, 1938.

Brewer, E. D.: On the limit of skin viability. *Med. Rec.*, New York, 27: 483, 1882.

Briggs, R., and Jund, L.: Successful grafting of frozen and thawed mouse skin. *Anat. Rec.*, 89:25, 1944.

Carrel, A.: The preservation of tissues and its application in surgery. *J. Am. Med. Assoc.*, 59:523, 1912.

Enderlen, D.: Uber die Anheilung getrockneter und feucht aufbewahrter Hautlappen. *Deutsche Zeitschr. f. Chir.*, 48:1, 1898.

Filatov, V. P.: Healing of Keratitides and certain skin diseases by tissue transplantation. *Vestnik oftalm.*, 11:295, 1931 (cited by Strumia).

Girdner, J. H.: Skin grafting with grafts taken from dead subjects. *Med. Rec.*, New York, 20:119, 1881.

Hanks, J. H., and Wallace, R. F.: Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. and Med.*, 71:196, 1949.

Kubanyi, E.: Uber provisorische Aufbewahrung der zu transplantirenden Gewebe. *Archiv f. Klin. Chir.*, 161:502, 1930.

Lewis, S. R., Pomerat, C. M., and Ezell, D.: Human epidermal cells observed in tissue culture with phase-contrast microscopy. *Anat. Rec.*, 104:487, 1949.

Ljunggren, C. A.: The capacity of epithelium to survive in storage. *Deutsche Zeitschr. f. Chir.*, 47:608, 1898.

Marangoni, A. G.: An experimental study on refrigerated skin grafts stored in ten per-cent homologous serum. *Plastic and Recon. Surg.*, 6:425, 1950.

Mathews, D. N.: Storage of skin for autogenous grafts. *Lancet*, 1:775, 1945.

Mider, G. B., and Morton, J. J.: The effect of freezing in vitro on some transplantable mammalial tumors and on normal rat skin. *Amer. J. Cancer*, 35:502, 1939.

Pierce, E. C. II, Gross, R. E., Bill, A. H. Jr., and Merrill, K. Jr.: Tissue culture evaluation of the viability of blood vessels stored by refrigeration. *Amer. Surg.*, 129:333, 1949.

Strumia, M. M., and Hodge, C. L.: Frozen human skin grafts. *Amer. Surg.*, 121:860, 1945.

Webster, J. P.: Refrigerated skin grafts. *Amer. Surg.*, 120:431, 1944.

Wentscher, J.: Die Verwendung Konservierter Hautlappen bei der Transplantation nach Thiersch. *Berliner Klin. Wochenschr.*, 31:979, 1896.

Wentscher, J.: Ein weiterer Beitrag zur Uberlebensfahigkeit der menschlichen Epidermiszellen. *Deutsche Zeitschr. f. Chir.*, 70:21, 1903–4.

THE ACTION OF SEX HORMONES ON THE METABOLISM OF AMINO ACIDS IN THE PROSTATE*

by

JORGE AWAPARA

It is perhaps unusual to have chosen the prostate gland as an organ to investigate protein metabolism and the factors involved in the regulation of tissue growth by hormones. It is, however, an unusual organ in that its rate of growth is greatly dependent on testicular hormones to which the prostate responds by a rapid increase in weight. Such a state of rapid growth brought about under nearly physiological conditions offers an ideal situation to investigate the mechanisms whereby hormones influence metabolism. Furthermore, the prostate is a frequent site of cancer which responds to hormone therapy.

The recognition of these facts by Huggins (1945) and Guzman Barron and Huggins (1944, 1946), brought forth a series of investigations on the metabolism of the prostate. The present status of our knowledge on the biochemistry of the prostate gland is rudimentary and only a few observations have been recorded.

CARBOHYDRATE METABOLISM

The effect of orchiectomy and diethyl stilbesterol upon carbohydrate metabolism was investigated in isolated dog prostate. (Guzman Barron and Huggins, 1944). The oxidative phase of carbohydrate metabolism was found to be dependent on the male sex hormone, as indicated by the low oxygen uptake in the castrated group, as compared to the normal group. However, anaerobic glycolysis remained undisturbed. The nature of this inhibitory effect could be, of course, of a generalized type and not a specific one.

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The synthesis of citrate was also investigated (Guzman Barron and Huggins, 1946), and found to proceed at a rate similar to that obtained in the heart of the rat. No utilization of citrate or ∝-ketoglutarate was observed and only a small but constant amount of pyruvate was utilized.

The effect of androgen and estrogen on the succinic dehydrogenase and cytochrome oxidase of the prostate was studied by Davis, Meyer, and McShan (1949), who found a similar relationship between the male sex hormone and enzymatic activity.

These experiments point clearly to a generalized diminution of metabolic activity in the absence of testicular hormone rather than to any specific effects. The loss of weight in the prostate resulting from castration indicates, of course, a loss of protoplasm, hence of proteins in general. It is already known, from studies on protein deficient rats, that enzymatic activity in the liver decreases rapidly, and that this loss of activity does not necessarily occur after a drastic loss of protein, but it can occur soon after the deficiency has developed (Litwack, Williams, Feigelson and Elvehjem, 1950).

Citric Acid in the Prostate:—The occurrence of large amounts of citric acid in the prostate has been reported on several occasions (Dickens, 1941; Humphrey and Mann, 1948 and 1949; Mann, 1949). This substance, which also appears in large amounts in the seminal plasma, is formed in most animal species in the prostate, by a mechanism which probably involves the condensation of oxaloacetic acid and pyruvate. The enzyme aconitase which converts cisaconitic acid to citric acid has been measured in dog prostate and human hypertrophic adenoma (Guzman Barron and Huggins, 1946). Values reported fall within the range of values found for kidney, liver, and other active organs of the rat.

The absence of enzymes capable of utilizing citrate and ∝-ketoglutarate seems to indicate that the Krebs cycle does not operate in this organ, and also explains this unusual accumulations of citrate. It may be seen from Table I that the prostate is indeed very rich in citrate as compared to other organs.

TABLE I

Citric Acid Content of Organs

Rabbit:		
	Liver	2.8 mg/100 gm
	Kidney	2.5 mg/100 gm
	Brain	6.0 mg/100 gm
Guinea Pig:		
	Liver	1.6 mg/100 gm
	Kidney	3.9 mg/100 gm
	Brain	3.8 mg/100 gm
	Testis	11.5 mg/100 gm
Rat:		
	Seminal Vescicle	39.0 mg/100 gm
	Median Prostate	60.0 mg/100 gm
	Dorsal Prostate	20.0 mg/100 gm
	Ventral Prostate	120.0 mg/100 mg

Humphrey and Mann (1948) have shown that there is a close relationship between the citric acid formation in the male reproductive system and the function of the testicular hormone. Following castration, there is a remarkable fall in the citrate content of the prostate and other accessory organs.

AMINO ACID METABOLISM

In contrast to carbohydrate metabolism, the metabolism of amino acids has not been investigated at all, except for a few experiments reported by Guzman Barron and Huggins (1946) on transaminations in dog prostate. The reaction Glutamate + Oxaloacetate $\rightleftharpoons$ Aspartate + α-ketoglutarate reached equilibrium at the end of a one-hour period. They postulated that this active transamination gave rise to oxaloacetate which is obligatory for the synthesis of citrate. The authors do not explain, however, how the α-ketoglutaric acid is formed, since it cannot be produced from citrate as they had previously demonstrated.

We took interest in the prostate gland as a means to study the relationship which may exist between testicular hormones and amino acid metabolism. Some time ago, experiments of a qualitative nature were performed in our laboratory to ascertain this relationship (Marvin and Awapara, 1949).

These experiments revealed that a decrease in the amounts and number of free amino acids determinable by paper chromatography was associated with prostatic atrophy after castration. Furthermore, replacement therapy in the castrated animal was followed by a progressive increase toward normal. It seems that the same relationship exists for amino acids as it does for other substrates. It is difficult to explain this drop of concentration in the free amino acids of the prostate in terms of reduced protein synthesis, or protein breakdown. It may be easier explained in terms of active amino acid catabolism. With such scant data, of course, all explanations must remain speculative. The experiments were performed in whole prostate, and no distinction was made between the various parts, because the atrophied condition of the gland made it almost impossible to attempt dissection. It has been learned since, that the ventral lobe of the prostate exhibits an entirely different amino acid distribution pattern than the dorsal portion.

Free Amino Acids in the Prostate:—Since it was demonstrated previously that the dorsal prostate is different from the ventral portion, all experiments were carried out separately after dissection of the gland. The free amino acid distribution pattern of both parts was determined by means of paper chromatography. The dorsal lobe resembles other organs of the rat, but the ventral portion is in a class by itself. The author has investigated by the same means every organ of the rat and found that most organs have a distinct pattern of distribution of free amino acids. However, the similarities were greater than the differences (Awapara, Landua and Fuerst, 1950). The ventral prostate of the rat is unique in that it possesses practically every known amino acid and some amino compounds commonly occurring, such as taurine, glutamine, glutathione, and aminoethylphosphoric ester. Not only is the number of amino compounds greater than in any other organ, but the concentration of each component is also higher than in most organs.

Effect of Sex Hormones and Castration on the Free Amino Acids of the Prostate:—In an attempt to find out whether sex hormones have an effect on the pattern of amino acid distribution in the prostate, mature rats were given various treatments

and the prostates examined. Castrated rats were sacrificed six days afer the operation, when the prostate gland had lost considerable weight. Other groups were treated as follows: 1 mg Testosterone propionate per day for seven days, 5 mgs Progesterone per day for seven days, and 5 γ Estradiol acetate per day for seven days. After the treatment, the animals were sacrificed, the prostates removed, dissected and analyzed for free amino acids by means of paper chromatography. The results were unimpressive, except in the castrated animals which exhibited a marked drop in the concentration of nearly all the amino acids. The drop in concentration did not seem to be equal for all amino acids; aspartic and glutamic acids seemed to remain almost at the same level than in the normal group. No quantitative measurement of the amino acids was carried out since at this stage only qualitative changes were being sought.

Transaminations in the Prostate:—Having demonstrated that the ventral prostate possesses a high concentration of free amino acids which seems to be regulated by testicular hormones, it remains to ascertain the site of action of testosterone and perhaps determine the mechanism which maintains such high levels of free amino acids in the prostate. It was observed that the distribution of citric acid in the accessory sex organs of the rat runs parallel to the distribution of amino acids. The ventral lobe is rich in citric acid (120 mg per 100) as compared to the dorsal lobe (20 mg per 100). The same partition seems to exist for free amino acids in the ventral and dorsal lobes. This observation seems to indicate that there may be a direct relationship between citric acid synthesis and accumulation of free amino acids. This relationship could exist in view of the high demand for oxaloacetic acid which is an obligatory component for citrate formation. Guzman Barron and Huggins (1946) have previously demonstrated that, at least in the dog prostate, the Krebs cycle does not operate since they could not demonstrate citric acid, nor α-ketoglutaric acid utilization. Under these circumstances, one may assume that the Krebs cycle is blocked at the citrate stage, and hence no α-ketoglutaric acid, nor indeed oxaloacetic acid may be formed by this

mechanism. The same investigators have shown active transamination in the prostate and postulated that the oxaloacetic acid required for this reaction was continuously supplied by transamination of aspartic acid. Of course, one must still find a source for ∝-ketoglutaric acid, which is needed in this reaction.

This situation might be easier understood if one assumes that the oxaloacetic acid, from whichever source, is immediately utilized in the synthesis of citrate which accumulates in the prostate and which is secreted in large amounts into the seminal plasma. At this stage, all the pyruvic and oxaloacetic acids are utilized and no ∝-ketoglutaric acid can be formed. This situation would create a deficiency of keto acids, which are necessary for transaminations, and perhaps cause an accumulation of amino acids. This postulate was tested experimentally and though unequivocal proof is lacking, the evidence points clearly to the existence of active transaminases in the prostate.

Ventral prostate homogenates were incubated at pH 7.5 with ∝-ketoglutaric acid under anaerobic conditions. At the end of one hour of incubation, the homogenates were analyzed for free amino acids. The decrease in amino acids and the increase in glutamic acid are discernible in the chromatograms shown in Figs. 1 and 2. It is not at all surprising to observe that all amino acids have reacted in what appears to be a transamination mechanism. The scope of the transamination reaction has now been extended to a great variety of amino acids, as was demonstrated in other organs (Cammarata and Cohen, 1950). Semiquantitative data indicates that there is a drop of at least 50 per cent for most amino acids and a corresponding increase in glutamic acid (Table II).

Discussion

The fate of the intracellular amino acids in the prostate seems to depend upon the state of the gland. The observations recorded in this paper are only of a qualitative nature, but some of the changes which occur are discernible and cannot be ignored; it is clear that the maintenance of high amino acid levels

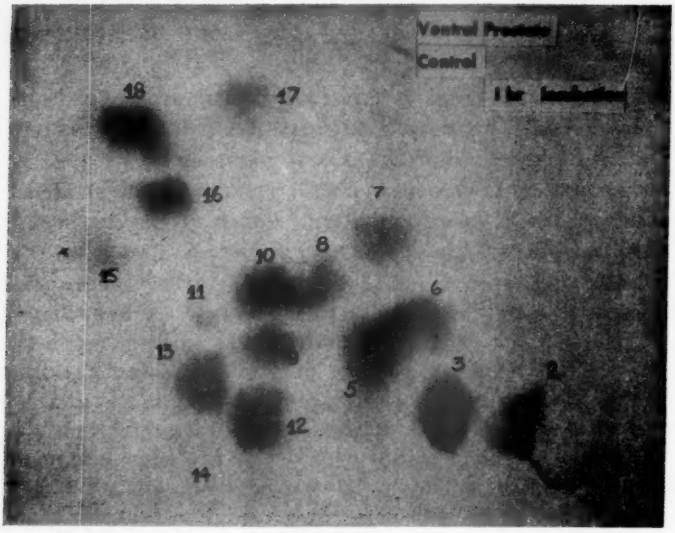

FIGURE 1

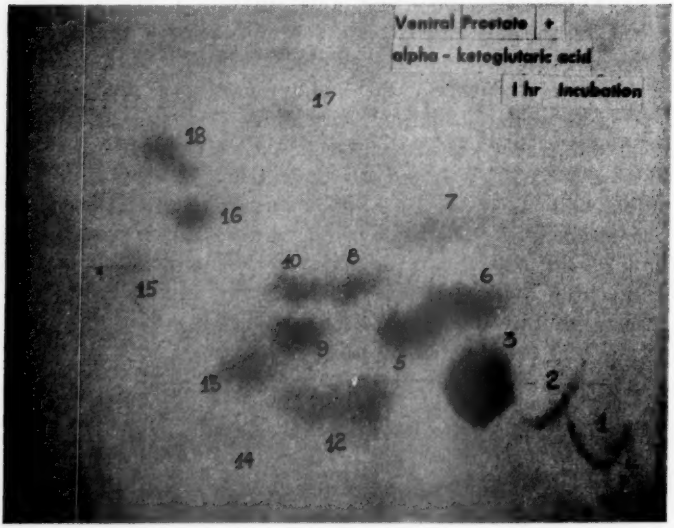

FIGURE 2

Two dimensional chromatograms of extracts from ventral prostate after the incubation with and without α-ketaglutaric acid. The following compounds are discerned: (1) glutathione, (2) aspartic acid, (3) glutamic acid, (5) glycine, (6) serine, (7) taurine, (8) threonine, (9) glutamine, (10) alanine, (12) lysine, (13) arginine, (14) unidentified, (15) proline, (16) valine, (17) tyrosine, (18) mixture of methionine, leucine, and isoleucine.

TABLE II

Transaminations in the Ventral Prostate

Amino Acid	Control μM/gm	α-Ketoglutaric μM/gm	Net Change
Aspartic	2.9	1.4	— 1.5
Glutamic	8.2	16.3	+ 8.1
Glycine	6.2	3.8	— 2.4
Serine	3.5	2.5	— 1.0
Threonine	1.4	0.9	— 0.5
Glutamine	1.7	1.8	+ 0.1
Alanine	4.2	1.4	— 2.8
Arginine	3.9	2.6	— 1.3
Proline	1.1	0.8	— 0.3
Meth-Val.	3.3	1.4	— 1.9
Phen-Leu.	5.3	1.9	— 3.4
Tyrosine	2.4	1.4	— 1.0

in the prostate is regulated by androgens and that in the castrated animal this high level cannot be maintained. Furthermore, it is of interest to observe the wide scope of the transamination reaction which appears to be extended to nearly all amino acids at least in the ventral prostate.

Attempts to explain the high level of free amino acids lead to several possibilities: (1) they may result from rapid protein breakdown, (2) rapid absorption from the blood, (3) slow protein synthesis, and (4) slow amino acid catabolism. It is conceivable that the concentration of free amino acids in the prostate or in any other tissue is the resulting balance of several of those pathways. Diagrammatically, this situation is shown in Fig. 3. As an oversimplification of the catabolic pathway of amino acids, the transamination reaction has been placed in this diagram as the only reaction which will lead to deamination, subsequent glycogenesis and urea formation. The choice of this sequence was made on the basis of Braunstein's (1947) views on the role of transamination in amino acid metabolism. According to this view, deamination of most amino acids by transaminases leads to glutamic acid formation which is rapidly deaminated by glutamic acid dehydrogenase, or perhaps participates directly in the formation of urea. The large quantities of free glutamic acid and its derivatives in animal tissue seem to support this view.

The anabolic action of androgens could perhaps be explained in terms of the above sequence of reactions. It could be possible

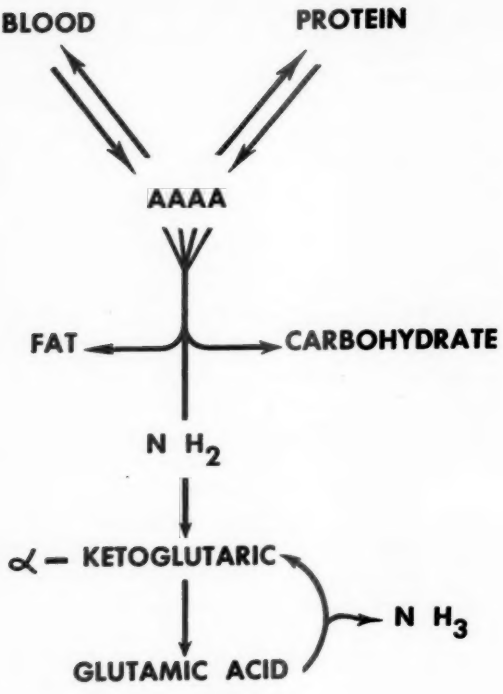

FIGURE 3

for androgens to inhibit the transamination reaction, thus blocking any further degradation of free amino acids which could then be used for protein synthesis. The same type of regulation would apply to other hormones involved in the regulation of protein synthesis and in tissues other than those of the prostate gland.

BIBLIOGRAPHY

Awapara, J., Landua, A. J., and Fuerst, R.: "The Distribution of Free Amino Acids in Organs of the Rat", *Biochim. et Biophys. Acta*, 5:457–462, 1950.

Braunstein, A. E.: *Advances in Protein Chemistry*, Vol. III, Academic Press, 1947.

Cammarata, P. S., and Cohen, P. P.: "The Scope of the Transamination Reaction in Animal Tissues", *J. Biol. Chem.*, 187:439–452, 1950.

Davis, J. S., Meyer, R. K., and McShan, W. H.: "Effect of Androgen and Estrogen on Succinic Dehydrogenase and Cytochrome Oxidase of Prostate and Seminal Vescicle", *Endocrinology*, 44:1–5, 1949.

Dickens, F.: "The Citric Acid Content of Animal Tissues with Reference to its Occurrence in Bone and Tumor", *Biochem. J.*, 35:1011–1015, 1941.

Guzman Barron, E. S., and Huggins, C.: "The Metabolism of Isolated Prostatic Tissue", *J. Urol.*, 51:630–634, 1944.

Guzman Barron, E. S., and Huggins, C.: "The Metabolism of the Prostate: —Transamination and Citric Acid", *J. Urol.*, 55:385–390, 1946.

Guzman Barron, E. S., and Huggins, C.: "The Citric Acid and Aconitase Content of the Prostate", *Proc. Soc. Exp. Biol. Med.*, 62:195–196, 1946.

Huggins, C.: "The Physiology of the Prostate Gland", *Physiol. Rev.*, 25: 281–295, 1945.

Humphrey, G. F., and Mann, T.: "Citric Acid in Semen", *Nature*, 161:352, 1948.

Humphrey, G. F., and Mann, T.: "Studies on the Metabolism of Semen. Citric Acid in Semen", *Biochem. J.*, 44:97, 1949.

Litwack, G., Williams, J. N. Jr., Feigelson, P., and Elvehjem, C. A.: "Xanthine Oxidase and Liver Nitrogen Variation with Dietary Protein", *J. Biol. Chem.*, 187:605–611, 1950.

Mann, T.: "Metabolism of Semen", *Adv. in Enzymology*, 9:329, 1949.

Marvin, H. N., and Awapara, J.: "Effect of Androgen on Concentration of Certain Amino Acids in the Rat Prostate", *Proc. Soc. Exp. Biol. Med.*, 72:93–95, 1949.

ACCESSORY CORPORA LUTEA IN THE WILD NORWAY RAT*

OCTAVIA HALL†

The purpose of this communication is to report upon the occurrence in nature, of accessory corpora lutea in the wild Norway rat. Presence of these structures in the monkey was first described by Corner (1940) where their occurrence was noted as a deviation from the accepted picture of the anatomical changes during the reproductive cycles in the mammalian ovary. A recent review of the literature on the corpus luteum by Harrison (1948) mentions the occurrence of accessory, aberrant and other atypical forms of corpora lutea as having been reported in the macaque, the tree porcupine and the mare. Mossman and Judas (1949) report upon the frequent occurrence of accessory corpora lutea in the Canadian porcupine.

MATERIALS

Rats were caught in an ecologically uniform habitat over a continuous period of 13 months and brought to the laboratory for autopsy as described by Davis and Hall (1950).

For histological study of the ovaries, the organs were fixed in Bouin's fluid, stained *in toto* in cochineal, serially sectioned at 10 and 15 micra, mounted on 2 x 3 inch slides, counterstained on the slides with cotton blue and mounted in Canada balsam under plastic slide-covers. A total of 394 pairs of ovaries was thus prepared and serially studied microscopically.

OBSERVATIONS

While making a microscopic examination of the serial sections to note the number of sets of corpora lutea present and the number of corpora lutea in each set (Hall and Davis, 1950),

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†The Carter Physiological Laboratory, The University of Texas Medical Branch, Galveston, Texas.

we also noticed early in the course of this study structures which were composed of lutein cells that matched the appearance of the cells in the one or the other set of corpora lutea in that ovary. These structures were found to be accessory corpora lutea reported in the literature in a few isolated species.

Accessory corpora lutea are composed of true lutein cells with a large amount of cytoplasm which is homogeneous and deeply staining in the more recently luteinized cells (fig. 4), and with a larger number of fat vacuoles, where the fat globules had dissolved away in fixation, in cells luteinized for a longer time. In the relatively recent accessory corpora lutea, the central area shows lutein transformation of the cells, while a band of granulosa cells may still be apparent around the periphery of the structure (fig. 4). The walls of the accessory corpora lutea are made up of cells of the same morphological appearance as the corpora lutea they accompany. Connective tissue cells are rare in the newly luteinized structures but became progressively more abundant in the older accessory corpora lutea, as indeed, in the older corpora lutea.

In complete agreement with Corner's findings (1940) each set of accessory corpora lutea found in our material was composed of cell types identical to those of the corpora lutea to which they were satellites. They had the same morphological arrangement and the same degree of luteinization or retrogression as one or the other set of corpora lutea found in that ovary. Such accessory corpora lutea are formed from luteinization of unruptured follicles of all sizes (figs. 1 and 2). Ova in all stages of degeneration from those showing only slight deterioration to those almost completely disintegrated were seen trapped within the accessory corpora lutea. Most often, however, no evidence of ova was seen, presumably because these had degenerated beyond the point of recognition (figs. 3 and 4). In general, accessory corpora lutea were not localized in any part of the ovarian parenchyma but were found in both the cortical and medullary areas. They bore no particular spatial relationship to the corpora lutea which they accompanied. However those formed from Graafian follicles and containing a trapped ovum, were found uniformly to be in the cortical region and near the ovarian surface in our material.

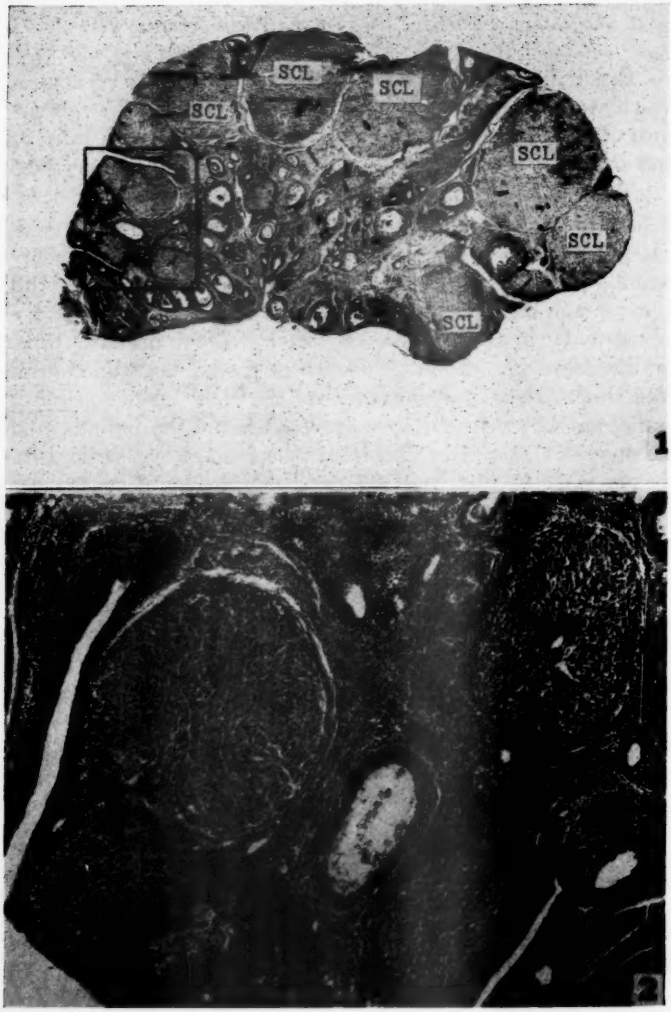


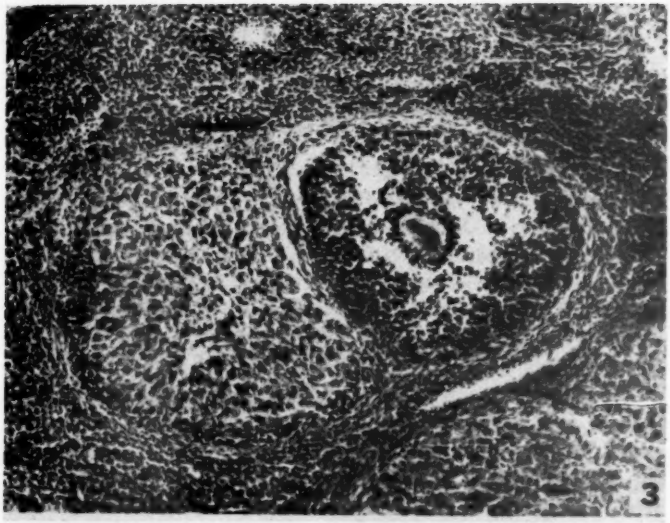
PLATE 1

Explanation of Figures

FIG. 1. Section through an ovary of a mature rat showing 6 new corpora lutea (SCL). The two smaller ones in the lower right hand corner might easily be mistaken for large accessory corpora lutea in a study of this solitary section. Serial examination however, shows that these assume the same proportions as the other 4 but at a different level of section. X 15.

FIG. 2. Insert from Fig. 1. X 75.

Four new accessory corpora lutea with the two on the right showing remnants of granulosa cells around the periphery.



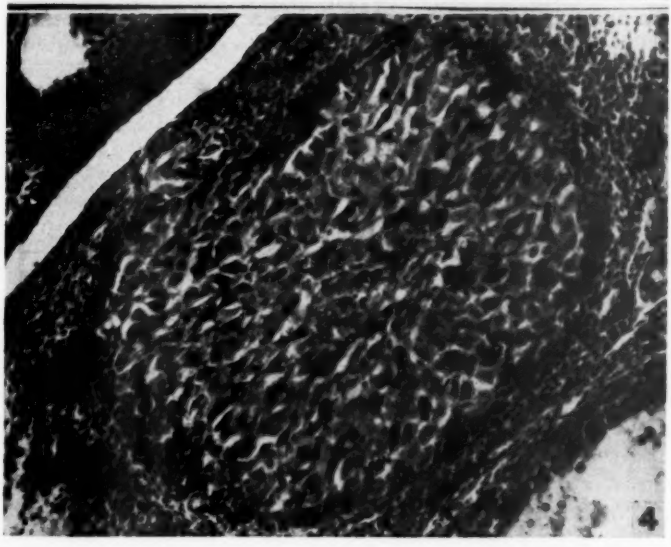


PLATE 2

FIG. 3. Two secondary follicles in close proximity. The one on the right is normal while the one on the left shows luteinization of its granulosa cells. X 150.

FIG. 4. Higher magnification (X 250) of a luteinized secondary follicle showing homogeneous deeply staining cytoplasm and a band of granulosa cells around the periphery. All evidence of an ovum has disappeared.

The size of the accessory corpora lutea ranged from that of a small secondary follicle (fig. 2) to that of a Graafian follicle about to ovulate. Indeed large Graafian follicles with trapped ova surrounded by luteinized granulosa cells were found. More typical, however, was the presence of some serous fluid, staining like liquor folliculi, in the middle or to the side of a Graafian follicle whose granulosa cells were totally luteinized. We feel that a later stage in the luteinization of such Graafian follicles would be characterized by the absence of ova or these pools of fluid, and histologically would be indistinguishable from corpora lutea, being easily mistaken for the latter.

Accessory corpora lutea occurred in 64.3 percent of those ovaries in which corpora lutea were found (Table I), and in every case they could be easily related to the one or the other set of corpora lutea seen in the ovary.

TABLE I

Accessory Corpora Lutea Found in Ovaries with Corpora Lutea	
No. of ovaries in this series	650
No. of ovaries recorded with only a "new"* set of accessory corpora lutea	314
No. of ovaries recorded with only an "old"† set of accessory corpora lutea	78
No. of ovaries recorded with "new" plus "old" sets of accessory corpora lutea	26
Percentage of ovaries with either "new" or "old" accessory corpora lutea	64.3

*Corresponding to a new set of corpora lutea.
†Corresponding to an older set of corpora lutea.

Accessory corpora lutea were found in pregnant as well as nonpregnant rats, in which the body weight ranged from 140 gms to 590 gms. There seemed to be no correlation between the presence of accessory corpora lutea and pregnancy, lactation, or pregnancy with lactation. Since no lutein tissue was observed in the 138 ovaries of immature females studied, it would appear that accessory corpora can only be formed under circumstances which also cause the development of corpora lutea in this species.

DISCUSSION

Histologic appearance of accessory corpora lutea suggests that they very probably possess the same endocrine function as

corpora lutea (Corner, 1940). Our studies indicate that such accessory corpora are of relatively common occurrence in adult wild Norway rats, showing an incidence of 64.3 percent. It is difficult to evaluate the functional significance of these structures, inasmuch as definitive studies elucidating their activity and importance have not been undertaken. Apparently luteinization of mature follicles, which in some cases contain entrapped ova, may be induced in the laboratory rat by appropriate treatment with gonadotrophins (Allen, 1942). It is tempting to assume that the anterior hypophysis, and possibly the placentae of the wild Norway rat behave somewhat hyperactively in comparison with those of the domestic strain, thus reproducing naturally those changes which may be induced experimentally by injection of gonadotrophins. The view that the accessory corpora lutea, like the corpora lutea, are under the same controlling mechanism is supported by the fact that the former do not occur in the absence of the latter, and that the accessories of each set of corpora lutea display developmental or retrogressive changes which closely paralleled those of the corpora of which they are the satellites.

Summary

Ovaries from 325 mature wild Norway rats possessing corpora lutea were serially sectioned and submitted to histologic examination. Of these 209, or 64.3 percent, showed the presence of accessory corpora lutea. Ovaries of an additional 69 immature rats with no corpora lutea present were similarly studied, but no accessory corpora lutea were found. The possible significance of these findings is discussed.

REFERENCES

Allen, Edgar: Physiology of the ovaries. Glandular physiology and therapy. *Am. Med. Assoc.*, Chicago, 143–167, 1942.

Corner, G. W.: Accessory corpora lutea in the ovary of the monkey Macaca rhesus. *Anales Fac. de med. de Montevideo*, 25:553–560, 1940.

Davis, David E., and O. Hall: The seasonal reproductive condition of female Norway rats in Baltimore, Maryland. *Physiol. Zool.*, 24:9–20, 1950.

Hall, O., and David E. Davis: Corpora lutea counts and their relation to the numbers of embryos in the wild Norway rat. *Texas Reports on Biology and Medicine*, 8:564–582, 1950.

Harrison, R. J.: The development and fate of the corpus luteum in the vertebrate series. *Biol. Rev.*, 23:296–331, 1948.

Mossman, H. W., and Ilse Judas: Accessory corpora lutea, lutein cell origin, and the ovarian cycle in the Canadian porcupine. *Am. Jour. Anat.*, 85:1–40, 1949.

ABSTRACT

Accessory corpora lutea in the wild Norway rat

Octavia Hall

Histological examination of serial sections of ovaries from 325 mature wild Norway rats showed the presence of accessory corpora lutea in 64 percent of the cases. Such structures were absent from ovaries of immature rats. A description of the accessory corpora lutea is given.

EVALUATION OF CHOLINE AND RELATED SUBSTANCES AS ANTITOXIC AGENTS TO NITROGEN MUSTARD *IN VIVO**

GORDON W. HOWE,† HORACE N. MARVIN, AND CHARLES L. SPURR

Anslow, *et al.* (1947) have reported several unsuccessful attempts to alter the LD50 of the Nitrogen Mustard, Methyl-bis B-Chloroethyl amine (HN_2) by the prophylactic or therapeutic administration of various agents. The importance of cyclic immonium degradation of the amino-B-chloroethyl group in enzyme inactivation and the cytotoxic activity of these compounds introduced by Golumbic (1946) is accepted. The cytotoxic effect as shown by Graef, *et al.* (1948) is specifically related to the B-Chloroethyl group since methyldiethanolamine is innocuous. It has been demonstrated by Barron, *et al.* (1948b) that there is a sharp decrease in oxygen uptake in cells of the hemopoietic system in the presence of small amounts of HN_2. Earlier work by Barron, *et al.* (1948a) detected an inhibition of choline oxidase and several other related enzymes by HN_2. Choline completely prevented the inhibitory action by HN_2, *in vitro;* whereas, dimethylethanolamina plus methionine was 93% effective, and dimethylethanolamine was only 81% as effective. They postulated that these compounds act as structural inhibitors to the reactive groups of enzyme protein moieties. The *in vivo* investigation of the potential protective action of choline and related substances on the hemopoietic system may, therefore, be of value in producing selective cytotoxic properties of HN_2 to various tissues. We have attempted to evaluate this by a study of the response of the hemopoietic system and survival to approximately LD50 doses.

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†Rosalie B. Hite Post-doctoral Fellow; present address: 180 Carroll Street, Paterson 1, N.J.

Procedure. Male rats used in these experiments were of the Sprague-Dawley strain varying in weight from 120–300 g. Animals were confined in cages in groups not larger than 5 and were observed from 20 to 28 days. Food and water were allowed *ad lib.* The compounds employed were diluted with 0.9% saline in the following concentrations: HN_2, 1 mg/cc, choline chloride, 2%, dimethylethanolamine and betaine hydrochloride, 10%, and ethanolamine, 5%. The dimethylethanolamine and ethanolamine were buffered to pH 7.3 with citric acid and the betaine hydrochloride was buffered to the same pH with sodium bicarbonate. In order to establish a suitable dose the LD50 of choline was determined by the standard procedure (Sollman, 1942). At the LD30[1] toxic symptoms first appeared. This was arbitrarily established as the quantity saturating free enzyme prosthetic groups.

Blood was obtained from the tail for determinations of the hemogram. Control counts were in agreement with the data of Thewlis and Meyer (1942) and Griffity and Ferris (1942). Hemoglobin was determined as acid hematin with a Klett-Summerson photoelectric colorimeter. Three daily control counts preceding the injection of test compounds and blood counts were continued daily except Sunday for 2 weeks. To evaluate the action of choline in the protection of the hemopoietic system, and on percentage fatality following HN_2, 4 control groups were used. The first group consisted of normal rats, the second received a single intravenous dose of choline (30 mg/kg), the third received 8 injections of choline (20 mg/kg) at 30 minute intervals, and the fourth group received HN_2 at slightly above the LD50. The *critical test group* received 2 injections of choline (20 mg/kg), an injection of HN_2, followed by 6 similar subsequent injections at 30 minute intervals. All injections were given intravenously. Ethanolamine, dimethylethanolamine, betaine and HN_2 were administered in a similar fashion and were observed for percentage fatality.

Results. Effect Upon Hemogram. The observations on the blood count following each of the above substances is summarized in Table I. The expected fall in leucocytes following HN_2

[1]LD30—Dose producing 30% fatality on the sigmoid curve.

TABLE I

Influence of Choline and Nitrogen Mustard on Hemogram of Rats

Average erythrocyte, hemoglobin and leucocyte counts in pretreatment period and at minimum level where significant change occurred. Mean values of the standard errors are corrected for small numbers.

Drug	Dose	No. Rats	RBC Millions	HGB g/100 cc	WBC
Control		10	7.90±.09	14.8±.3	19180±480
Choline	30 mg/kg	7	8.47±.81	14.2±.2	12310±570
Choline	20 mg/kg x 8	10	9.00±.22	17.6±.2	
			*6.88±.16	15.9±.3	20270±270
HN_2	0.5 mg/kg	7	8.6 ±.4	—	16680±1204
			7.9 ±.5		2936±985
Choline	20 mg/kg x 8	10	7.9 ±.89	—	15800±1211
HN_2	0.5 mg/kg		*6.5 ±.86		6675±1238

*Decline continued for 48–72 hrs. after last dose and then rapidly returned to original level.

developed and was not altered to a statistically significant degree by the preceding or simultaneous injection of the test compounds. It will be seen in Table II that there was no statisti-

TABLE II

Survival of Rats Receiving Single Injections of HN_2 With Choline or Derivatives in 8 Injections at 30 Min. Intervals.

HN_2 Given 30 Min. Following the Second Injection

Material	Dose mg/kg	No. of Rats Inj.	% Fatality
HN_2*	0.5	31	67.9
HN_2*	1.	21	61.9
HN_2* Choline†	0.5 20	26	46.2‡
HN_2 Choline	1. 20	15	73.3‡
HN_2 Dimethyl-ethanolamine†	1. 100	10	90. ‡
HN_2 Ethanolamine†	1. 10	5	60. ‡
HN_2 Betaine†	1. 1000.	8	50. ‡

*See explanation in text.

†Drugs given alone, in doses indicated, to groups of 5–10 rats produced only transient toxicity.

‡Differences between these figures and the percentage fatality with HN_2 alone are not statistically significant since the difference is less than three times the standard error of the difference of the 2 proportions.

cally significant protective action indicated by survival above the HN_2 LD50, since the percentage fatality difference was less than 3 times the standard error of the difference of the 2 proportions. Ethanolamine and betaine did not afford protection and dimethylethanolamine enhanced the lethal action of HN_2. It must be stated that during the first portion of this study a statistically sound determination of the toxicity of nitrogen mustard gave an LD50 of 0.5 mg/kg whereas later control groups resulted in an LD50 of 1.0 mg/kg. We are unable to explain this since rigidly standard conditions, including air conditioning, prevailed.

Toxicity of Choline and Related Substances. The LD100 of choline is 70–75 mg/kg body weight which is very close to the LD50 at 60–65 mg/kg. The LD100 of dimethylethanolamine is 150–180 mg/kg, that of ethanolamine is 20–25 mg/kg, and that of betaine is approximately 2 g/kg. The concentration and rate of injection must be rigidly controlled in order to maintain a constant LD50.

Discussion. The acute and chronic reactions of choline when administered orally and intraperitoneally have been reported in detail by Hodge *et al.* (1942, 1944, 1945) and by Neuman and Hodge (1945). Intravenous lethal doses in several species of animals other than the rat have been reported as varying from 35–60 mg/kg body weight (Dreyfus, 1920; Arai, 1922; Lohmann, 1907). One of the consistent toxic symptoms from choline, *but not the other derivatives studied,* includes red tears. Hodge (1945) demonstrated that this was due to erythrocytes which rapidly hemolyzed. Barnard (1943), confirmed by Totter,* has shown that these tears do not represent blood but are porphyrin containing droplets secreted by the Harderian glands.

The acute fall in the erythrocyte and hemoglobin levels are of interest and suggest two mechanisms. The cholinergic response may result in an increased blood volume, or intravascular hemolysis may have resulted from the choline. *In vitro* observations in this laboratory indicated that 10–30 gamma/cc hemolyze erythrocytes.

It has been established that choline disappears from the blood stream rapidly following intravenous injection (Hunt, 1915).

*Personal Communication.

This is accomplished by the action of choline oxidase without an increase in the choline content of various organs (Luecke and Pearson, 1945). A dose just sublethal may be repeated within a few minutes without toxic symptoms. In order to assure the physiological saturation of the enzyme systems with which choline and HN_2 might react, doses of approximately one-third LD50 of choline were used. It appears that these maximum doses resulted in no significant reduction in toxicity of the HN_2.

Clinical observations during the progress of this study support these conclusions. Choline chloride was administered at a constant intravenous rate over a 6 to 8 hour period with doses up to 150 mg/kg body weight. Nitrogen mustard was given 2 hours after starting choline. A standard course of 0.1 mg/kg on 4 consecutive days was given. We could not detect in a group of 4 cases, with lymphomas or leukemia, any alteration of the hemopoietic or clinical response. Minimal cholinergic symptoms were occasionally observed.

Summary

The intravenous lethal dose and LD50 of 2% choline chloride in the rat is 70–75 mg/kg and 60–65 mg/kg respectively. The intravenous lethal dose of 10% buffered betaine hydrochloride is approximately 2 g/kg; that of 10% buffered dimethylethanolamine is 150–180 mg/kg; and that of 5% buffered ethanolamine is 20–25 mg/kg. Choline injected intravenously in eight 20 mg/kg doses causes a significant decrease in the erythrocyte count and hemoglobin concentration lasting from 48–72 hours following injection. It has no other demonstrable effect on the hemogram. Choline and related substances cause no permanent toxic effect. Choline *in vivo* affords no protective action to methyl bis-B-chloroethylamine either in the hemogram or on the survival time. Dimethylethanolamine, betaine hydrochloride and ethanolamine have little effect on the toxic action of methyl bis-B-chloroethylamine.

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REFERENCES

Anslow, W. P., Jr., D. A. Karnofsky, Val B. Jager, and H. W. Smith: The Toxicity and Pharmacological Action of the Nitrogen Mustards and Certain Related Compounds. *J. Pharm. Exp. Therap.*, 91:224–235, 1947.

Arai, K.: Cholin als Hormon der Darmbewegung VI Mitteilung. *Arch. Ges. Physiol.*, 193:359–395, 1922.

Barnard, R. D.: Cholinergic Porphyrin Lacrymation and Paradoxical Mydriasis in the Rat. Possible Heme Nature of Choline Esterase. *Proc. Soc. Exp. Biol. and Med.*, 54:254–258, 1943.

Barron, E. S. G, G. R. Bartlett, and Z. B. Miller: The Effect of Nitrogen Mustards on Enzymes and Tissue Metabolism I. The Effect on Enzymes. *J. Exp. Med.*, 87:489–501, 1948a.

Barron, E. S. G., G. R. Bartlett, Z. B. Miller, J. Meyer, and J. E. Seegmiller: The Effect of Nitrogen Mustards on Enzyes and Tissue Metabolism II. The Effect on Tissue Metabolism. *J. Exp. Med.*, 87:503–519, 1948b.

Dreyfus, L.: De la toxicité des ptomaines. *C. R. S. B.*, 83:481–483, 1920.

Golumbic, C., J. S. Fruton, and M. Bergmann: Chemical Reactions of the Nitrogen Mustard Gasses I. The Transformations of Methyl-Bis (B-Chloro-Ethyl) Amine in Water. *J. Org. Chem.*, 11:518–535, 1946.

Graef, I., D. A. Karnofsky, V. B. Jager, B. Krichesky, and H. W. Smith: The Clinical and Pathologic Effects of Nitrogen and Sulfur Mustards in Laboratory Animals. *Am. J. Path.*, 24:1–47, 1948.

Griffity, J. Q., Jr., and E. J. Ferris: *The Rat in Laboratory Investigation.* J. B. Lippincott Co., Philadelphia, pp. 356, 1942.

Hodge, H. C., and M. R. Goldstein: The Acute Toxicity of Choline Hydrochloride in Mice and Rats. *Proc. Soc. Exp. Biol. and Med.*, 51:281–282, 1942.

Hodge, H. C.: Acute Toxicity of Choline Hydrochloride Administered Intraperitoneally to Rats. *Proc. Soc. Exp. Biol. and Med.*, 57:26–28, 1944.

Hodge, H. C.: Chronic Oral Toxicity of Choline Chloride in Rats. *Proc. Soc. Exp. Biol. and Med.*, 58:212–215, 1945.

Hunt, R.: A Physiological Test for Cholin and Some of Its Applications. *J. Pharm. and Exp. Therap.*, 7:301–337, 1915.

Luecke, R. W., and P. B. Pearson: Effect of Ingestion of Excessive Quantities of Choline on Amount in Tissues and Urine. *J. Biol. Chem.*, 158: 561–566, 1945.

Lohmann, A.: Cholin die den Blutdruck erniedrigende Substanz der Nebenniere. *Arch. Ges. Physiol.*, 118:215–227, 1907.

Neuman, M. W., and H. C. Hodge: Acute Toxicity of Choline Chloride Administered Orally to Rats. *Proc. Soc. Exp. Biol. and Med.*, 58:87–88, 1945.

Sollman, T.: *A Manual of Pharmacology.* W. B. Saunders Co., Philadelphia, 1942.

Thewlis, E. W., and O. O. Meyer: The Blood Count of Normal White Rats. *Anat. Rec.*, 82:115–125, 1942.

A STUDY OF NINETY-ONE CHILDREN WITH RHEUMATIC FEVER IN THE GULF COAST AREA OF TEXAS

HOWARD E. LEBUS AND THEO C. PANOS

Heyer and coworkers (1950) reported the variations in incidence of rheumatic fever deaths in various parts of Texas. The present writers were somewhat surprised to find such a small number reported for the region of Galveston. The year of 1946 had been selected by Heyer, *et al.*, and it so happens that during this particular year there was only one death from rheumatic fever on the pediatric service at The University of Texas Medical Branch.

Actually numerous investigations have been made regarding the incidence of rheumatic fever in various sections of the United States. Those pertaining to the Gulf Coast area (Decherd, 1943; Herrmann, Decherd, Ruskin, 1942; Stone and Vanzant, 1941; Schwab, Schulze, 1931) have emphasized the relative infrequency of the disease in individuals native to that region.

The accurate estimation of the prevalence of rheumatic fever is difficult to determine, inasmuch as this is not a reportable disease. None of the methods ordinarily used is ideal in every respect. Analysis of autopsy records is obviously unsatisfactory since only the fatal cases are included. The study of the number of hospital admissions provides an index of prevalence, but this is so only if the hospitals are closely comparable as to type, source of patients and available facilities in the community.

It is the purpose of the present study to report the results of a survey which was designed to ascertain the character and significance of the rheumatic fever problem in children in the Gulf Coast area of Texas.

MATERIALS AND METHODS

The records of all children with rheumatic fever admitted to the Pediatric Service of The University of Texas Medical

*From the Department of Pediatrics, The University of Texas Medical Branch, Galveston. Received for publication September 10, 1951.

Branch from January 1, 1945, to January 1, 1950, were analyzed in detail. Cases in which the diagnosis was doubtful or only suspected were not included. The great majority of patients admitted to the University Hospital are referred by practicing physicians throughout the state, but because of the location of the hospital in Galveston, there is a natural tendency for the Gulf Coast area to be represented preponderantly.

Results

Incidence: During the five year period mentioned, there were 3162 admissions to the pediatric service. This figure does not include newborns, children suffering from orthopedic disorders, or a few children who were admitted directly to the surgery service because of emergency conditions. A definite diagnosis of rheumatic fever was made in 91 cases or 2.87 percent of the total admissions. When those children who were admitted on more than one occasion were included there were 111 admissions because of rheumatic fever. Comparable data from similar institutions (*i.e.*, teaching hospitals reporting pediatric admissions) are presented in Table 1. It appears that rheumatic fever is as prevalent in the area served by The University of Texas Children's Hospital as in other areas of the country.

Table I

Percentage Hospital Admissions for Rheumatic Fever

San Francisco (Christie, 1950)	2.7
Nashville (Koenig, 1950)	2.8
New Haven* (Paul, 1941)	2.7
Dallas (Fashena, 1943)	0.8
Galveston	2.8

*Based on medical and pediatric admissions.

Geographic distribution: Reference to the community of referral as indicated diagrammatically in Figure 1 disclosed that about three-fourths (74 percent) of the patients were residents

of a belt less than 100 miles from the Gulf of Mexico and three-fifths (59 percent) originated from communities less than 50 miles inland (Figure 1). In addition, only 2 of the 91 patients

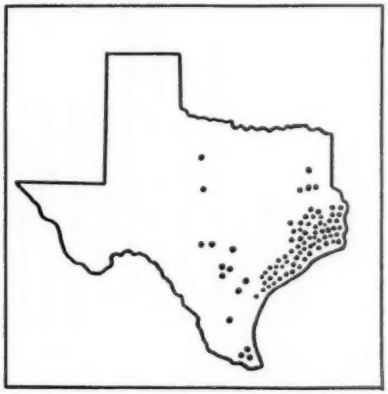

Fig.1 Geographical Incidence

experienced their initial attack of rheumatic fever while living outside the state. This is in sharp contrast to the findings of Decherd and Herrmann (1943) who made a similar type of study which included both children and adults. At the time of the initial attack, almost half of their patients were living outside the state of Texas.

Age, Sex and Race: The age at onset was between 6 and 12 years in 90 percent of the patients, peak incidence being at 8 and 9 years(Figure 2). Males comprised 45 percent of the total number of patients, females 55 percent. There were 34 percent negro and 66 percent white subjects.

Type of onset: The onset was acute in 63 percent and insidious in 37 percent. A definite history of a respiratory tract infection one–three weeks prior to onset was obtained in only 18 percent of the patients. This appeared to be a rather surprising finding in view of the high incidence of antecedent infections usually found in patients with rheumatic fever.

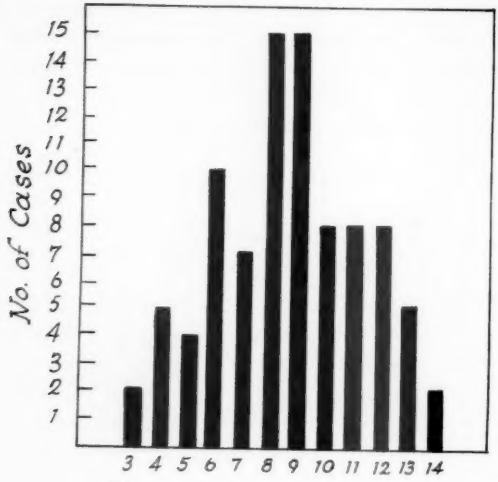

Fig. 2. Age at Onset

Familial incidence: A positive family history of polyarthritis, "leakage of the heart" or other evidences of rheumatic infection was obtained in only 15 percent of the patients.

Manifestations: Data concerning important symptoms and physical findings are summarized in Table 2.

Constitutional symptoms: Fever, easy fatiguability, malaise and anorexia were mentioned as symptoms in 88 percent of the patients; however, definite weight loss had occurred in only 23 percent and epistaxis in 18 percent.

Joint or muscle pain was the primary complaint in 79 percent of the patients. Overt evidence of arthritis such as redness, swelling and tenderness was present in 57 percent. Incidentally, abdominal pain was a prominent symptom in 31 percent of the patients.

Carditis: The relative frequency of the various cardiac findings is summarized in Table II. Systolic murmurs were present in 85 percent of the patients. Accompanying diastolic apical murmurs were present in 33 percent. Cardiac enlargement was found in 46 percent. Signs of failure were present in 23 percent and 9 percent had evidence of pericarditis.

TABLE II

Percentage Occurrence of Prominent Manifestations

Constitutional Symptoms	88
Arthritis	57
Joint and/or Muscle Pain	79
Carditis	
Murmurs: Systolic	85
Diastolic	33
Cardiac Enlargement	46
Cardiac Failure	23
Pericarditis	9
Chorea	23
Subcutaneous Nodules	6
Abdominal Pain	31
Epistaxis	18

Subcutaneous fibroid nodules were found in 6 percent of the patients.

Signs compatible with the diagnosis of chorea minor were present in 23 percent of the children. This was the only manifestation in nine, while in the remaining twelve, there was associated carditis and/or arthritis.

Laboratory findings: The results of the various laboratory aids employed in assisting in making the diagnosis are summarized as follows:

Complete blood counts: A moderate leukocytosis during an acute episode was quite consistently found. Hemoglobin values were above 11 gm.% in 64 percent of the patients, between 9 and 11 gm.% in 26 percent and below 9 gm.% in 10 percent. These values do not vary greatly from those in most of the patients admitted to the Children's Hospital.

Erythrocyte sedimentation rate: The erythrocyte sedimentation rate was greater than 30 mm/hr in 70 percent of the patients and greater than 20 mm/hr in 78%. In chorea without evidence of arthritis or carditis, values below 20 mm/hr were found in all except two patients, and one of these had an acute upper respiratory infection. During cardiac failure, the rate frequently was either normal or lower than normal, returning to high levels after compensation had been effected.

Bacteriologic studies: Repeated throat and blood cultures which were obtained in the majority of patients disclosed that beta hemolytic streptococci were present in only 16 percent of

the patients. Blood cultures were negative except in three patients who had subacute bacterial endocarditis.

Antistreptolysin titers: Routine determinations were performed until about January, 1949, at which time the procedure was abandoned because of the variable results obtained, and it was felt that this determination did not assist diagnostically nor in following the course of the disease.

Roentgenographic studies: The six-foot posterior-anterior projection films, which were taken periodically, revealed generalized cardiac enlargement in 32 percent of the patients and excessive straightening or fullness of the left upper cardiac border in an additional 32 percent. The right anterior oblique projection films revealed encroachment upon the esophagus by an enlarged left auricle in 41 percent of the patients. There was a direct and close correlation between severity of the disease and the presence of one or all of these roentgenologic abnormalities.

Electrocardiographic studies: Prolongation of the auriculoventricular conduction time was found in 35 percent of the patients. There were only eight tracings from as many patients in which abnormalities in rhythm or changes in the ventricular complex were found.

Mortality: During this five year period there were 11 deaths among the 91 children. Nine of these succumbed during their first admission. Five of the patients died during the initial attack. Rheumatic fever accounted for 4.8 percent of the total pediatric deaths during the five year period.

Discussion

The relative frequency of hospital admissions because of rheumatic fever throughout the country varies greatly. One reason for this is the difference in types of institutions, particularly in relation to the number of children admitted. It is rather remarkable that the percentage of hospital admissions because of rheumatic fever in New Haven (Paul, 1941), San Francisco (Christie, 1936), Nashville (Koenig, Weaver, Childers, Woods, 1950) and Galveston was very similar. Reports from Cincinnati (Wedum and Wedum, 1944) and Philadelphia (Hedley, 1940) disclosed definitely lower values of 0.52 percent

and 0.68 percent respectively. It so happens that for these latter studies the percentage of rheumatic fever admissions was based on total hospital admissions. If one considers total hospital admissions as a basis for calculation then the figure reported from New Haven by Paul, 1941 (2.7%) would drop to approximately the same range as reported from Cincinnati and Philadelphia. Decherd and Herrmann in 1943 reported the incidence to be 0.62 percent for the region of Galveston, but their data were based on total hospital admissions.

Fashena (1943) stated that the occurrence of rheumatic heart disease in the population in the region of Dallas is not unlike that reported from areas in which rheumatic fever is considered to be common. She pointed out that the 1941 death rate from rheumatic fever in Texas school age children was not strikingly less than that in the so-called rheumatic fever states. For Dallas she reported an incidence of only 0.8 percent admissions because of rheumatic fever, yet she based her data in relation to pediatric patients only. It should be pointed out that her report included out-patient as well as in-patient material, whereas in our study we included only those children who were admitted to the hospital.

Although there were eleven deaths due to acute rheumatic fever on the Pediatric Service of The University of Texas Medical Branch during the five year period studied, only one death occurred in 1946, and this was the year selected by Heyer, *et al.* (1950) for their study. It is evident that the record of this death was overlooked; however, had there been even two or three deaths reported from our institution the conclusions reached by their study would not have been altered.

The incidence of cardiac failure was about the same in our series as reported by others. The number of children who suffered from cardiac decompensation during their initial attack was surprisingly high. Seven children, all female subjects, were in this category and five succumbed during the first attack.

Insofar as one may ascertain from the data presented in our study, the nature of an attack of rheumatic fever in our patients regarding symptomatology, physical signs, and laboratory abnormalities is similar to that usually ascribed to this important disease of childhood.

Summary and Conclusions

Rheumatic fever constituted 2.8 percent of the total pediatric admissions to The University of Texas Medical Branch Hospitals between January 1, 1945, and January 1, 1950. This figure is comparable to that reported from similar institutions in other sections of the country.

The salient clinical manifestations have been summarized, along with laboratory studies which were used as aids in diagnosis.

BIBLIOGRAPHY

Christie, Amos: Rheumatic Fever in Northern California. *Am. Ht. J.* 12:153, 1936.

Decherd, G. M., Jr., and G. R. Herrmann: Rheumatic Heart Disease in Texas. *Tex. St. J. Med.*, 39:229–231, 1943.

Fashena, G.: Incidence of Rheumatic Fever in Texas with Particular Reference to the Dallas Area. *Tex. St. J. Med.*, 39:474–475, 1943.

Hedley, O. F.: Public Health Aspects of Rheumatic Heart Disease. *Public Health Reports*, 55:1,599, 1940.

Herrmann, G. R., G. M. Decherd, and A. Ruskin: Heart Disease in Texas. Public Health Aspects. *Tex. St. J. Med.*, 38:483, 1942.

Heyer, Howard E., Arild E. Hansen, Don W. Chapman, W. B. Adamson, and George W. Parsons: Deaths from Acute Rheumatic Fever in Texas: Relationship to Mean January Temperature. *Am. J. Med. Sc.*, 219:40–47, January, 1950.

Koenig, Leonard J., Thomas S. Weaver, Ada Childers, and Helen S. Woods: Rheumatic Fever in Tennessee. *So. Med. J.*, 43:199, 1950.

Paul, John R.: Rheumatic Fever in New Haven. Science Press Printing Co., Lancaster, Pa., 1941.

Schwab, E. H. and V. E. Schulze: Incidence of Heart Disease and of Etiological Types in a Southern Dispensary. *Am. Ht. J.*, 7:223–224, 1931.

Stone, C. T., and F. R. Vanzant: Heart Disease as Seen in a Southern Clinic, Clin. and Path. Survey. *J.A.M.A.*, 89:1,473, 1927.

Wedum, Arnold G., and Bernice G. Wedum: Rheumatic Infection in Cincinnati Hospitals. *Am. J. Dis. Ch.*, 67:182, 1944.

EXPERIMENTAL ATTEMPTS TO TRANSMIT HODGKIN'S GRANULOMA*

P. O'B. MONTGOMERY† AND THOMAS B. FOARD

Pathologically and clinically, Hodgkin's disease stands as a link between known virus diseases and pure neoplastic disorders. A great many workers have attempted to isolate a virus from the lesion, or to transmit the disease to experimental animals. To date all such attempts have met with failure. The purpose of this paper is to record further attempts, in which several different methods were used to transmit the disease to a variety of experimental animals.

Patients dying of Hodgkin's granuloma universally show widespread involvement of the obdominal and retroperitoneal lymph nodes. The most likely way for an infectious agent to enter these nodes is via the gastro-intestinal tract. The first series of experiments deals with an attempt to induce Hodgkin's granuloma in experimental animals by the oral feeding of Hodgkin's granuloma material obtained at autopsy from a patient dying of Hodgkin's disease.

Approximately 1000 grams of spleen, liver, and retroperitoneal lymph nodes were removed with sterile precautions, one hour after death, from a patient dying of proven Hodgkin's granuloma. Subsequent tissue examination of the postmortem material confirmed the diagnosis of Hodgkin's granuloma. The removed material was ground in a sterile Waring blender. Cultures of this material were sterile on Blood Agar plates in air and CO^2, in beef heart infusion broth, Brewer's liquid thioglycolate media, and on Litman's media and Sabouraud's media. After the cultures were taken an equal part of fifty percent glycerine and saline was added to the suspension. A sufficient

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*From the Veterans Administration Hospital, McKinney, Texas, and Southwestern Medical School of The University of Texas, Dallas, Texas. Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration. Received for publication September 15, 1951.

†Present address: Cancer Research Institute, New England Deaconess Hospital, Boston, Massachusetts.

amount of this mixture was passed through a Zeitz filter to obtain 100 c.c.'s of filtrate. The residue was saved separately. All samples were stored in the ice box at 34° F.

Twenty-four Wistar strain white rats, 24 stock mice and 24 stock guinea pigs were divided into three groups of eight rats, mice and guinea pigs each. An equal number of males and females were used, and no attempts to prevent breeding were made. All animals were fed standard Purina laboratory feed and water ad libitum. The rats ranged in age from one to one and one-half years, the mice from six months to one year, and the guinea pigs from four to six months.

Group A was fed 50 c.c. of whole tissue glycerine saline mixture per day for ten days. Group B was fed 20 c.c. of Zeitz filtered residue per day for 10 days. Group C was fed 10 c.c. of Zeitz filtrate per day for 10 days.

In Group A one rat died at the end of five months. Autopsy revealed a large reticulum cell sarcoma of the mesenteric and mediastinal lymph nodes (Bullock and Curtis, 1930). One mouse died at two weeks of generalized peritonitis. In Group C one rat died at four months with generalized peritonitis. Four mice died at one, two, three and four months respectively as a result of wounds inflicted by other mice.

In Group B one rat died at four months of generalized peritonitis. Three mice died of peritonitis at one day, one month and four months, respectively. Three guinea pigs died of peritonitis at two months, five months, and six months respectively.

All other animals in all three groups remained well for nine months. At this time they were sacrificed and complete autopsies performed. No lesions of Hodgkin's granuloma were encountered.

The second series of experiments using frozen tissue was inspired by the work of Gye and Craigie (1949). One hundred grams of surgically removed sterile lymph nodes were ground to a homogeneous semi-solid mass in a Waring blender. Tissue sections of the material removed before emulsification revealed the nodes to be diffusely replaced by Hodgkin's granuloma. Cultures of the emulsified material were sterile on blood agar aerobically and in 10% CO^2, beef heart broth infusion, Brewer's liquid thio-glycolate, Littman, Sabouraud's and Petrognani's

media. After the cultures were taken fifty milliliters of sterile distilled water were added to the emulsion. The entire mixture was placed in a round-bottom flask and shell-frozen in a dry ice and acetone mixture at minus 79 degrees centigrade. The frozen tissue was then partially lyophillized for two hours, during which time it was kept at minus 79 degrees centigrade. The dried mass was broken up into small pieces and kept at minus 79 degrees centigrade with dry ice during the course of the experiment. Daily a small amount of frozen tissue was mixed under sterile conditions with water in a mortar until thirty milliliters of a fine suspension of tissue in water was obtained. This material was used in all of the experiments described below.

Eight days after the initial freezing, four males and three females, Wistar strain, adult white rats, three male and four female adult stock white mice, two male and two female adult stock hamsters, one male adult white rabbit, one male and two female stock adult mongrel dogs, and three female and three male adult stock guinea pigs were injected with the tumor. One milliliter of material was injected intra-peritoneally for twenty-three consecutive days. One-half milliliter was injected into the right lung for six consecutive days. One-half milliliter was injected subcutaneously in the neck for five days on alternate days. One month after the initial freezing of the tumor these animals had 10 milliliters of tumor suspension per day added to their drinking water for ten consecutive days. The animals were fasted for water, and all the water was taken within one hour after it was offered. In this group of animals one rat died at the end of one month of generalized peritonitis. One rat died at the end of two months of reticulum cell sarcoma involving the mesenteric and mediastinal lymph nodes. Five days before death this animal showed progressive paralysis of the hind limbs. One dog died of distemper at two months. All other animals in this group remained well for seven months, at the end of which time all animals were autopsied. No lesions of Hodgkin's disease were encountered in any animal.

Twelve days after the initial freezing of the tumor two four-month old male O.I.C. hog litter mates were subjected to laparotomy, and 5 milliliters of tumor injected directly into the splenic

pulp. These two animals and a male litter mate were then given intraperitoneal and bilateral subcutaneous neck injections of 5 milliliters of tumor suspension for fourteen consecutive days. One month later the pig not operated upon was fed two hunderd and fifty grams of whole sterile Hodgkin's granulomatous lymph nodes. These nodes were obtained at autopsy from a patient dying of Hodgkin's granuloma. All three pigs remained well. At the end of seven months they were sacrificed and complete autopsies failed to show any lesions of Hodgkin's granuloma.

One month after the original freezing of the tumor, one adult male spider monkey was injected and fed the tumor suspension. Five milliliters of the tumor suspension was injected intraperitoneally and subcutaneously in the neck for five consecutive days; and then intraperitoneally for six days. Three days were allowed to lapse between each of the latter injections. During one week of this time 10 milliliters of tumor suspension was added to the monkey's drinking water. He was fasted for water daily, and the water containing the tumor suspension was readily taken when offered. The monkey remained well for seven months at which time he was sacrificed and a complete autopsy performed. No lesions of Hodgkin's disease were encountered.

Two months after the initial freezing of the tumor, six male and six femae young white rats, descendants of the originally injected rats, were subjected to fifty roentgens of total body irradiation. Three males and three females were kept as controls. The day following the irradiation the remaining six animals were injected intraperitoneally and subcutaneously in the neck with one milliliter of tumor for nine consecutive days. For five consecutive days, five milliliters of tumor suspension was added to their drinking water. The animals were fasted daily for water and readily drank the tumor-bearing water when it was offered. All of the animals in this group remained well for seven months, at which time they were sacrificed and complete autopsies performed. No lesions of Hodgkin's disease were encountered.

Discussion

Despite these and other reported failures to transmit Hodgkin's disease to experimental animals we do not feel that the subject has been exhausted. If the disease can be transmitted to animals a significant step in the final unmasking of its etiology will have been taken.

These experiments do not prove that Hodgkin's cannot be transmitted. They do, however, show that the methods used were inadequate to do so. The lack of a suitable host animal probably accounts for our failure to transmit the disease. With the exception of the report of Forbus (1946) of a granulomatous disease of swine resembling Hodgkin's disease, Hodgkin's disease is not known to exist in any animal except man; and, since man cannot be used in experimental attempts to transmit the disease, it may be that a suitable experimental animal will not be found. Nor is this a surprising finding, for the virus diseases already known are highly specific, not only for a given species, but for individuals within the species. The classical example is poliomyelitis; where thousands of unaffected carriers exist for every case. One should not, therefore, deny the virus etiology of Hodgkin's disease simply because it has not been successfully transmitted to experimental animals.

Summary

Unsuccessful attempts to transmit Hodgkin's granuloma to mice, rats, guinea pigs, hamsters, dogs, rabbits and a monkey by a variety of means are reported.

It is concluded that the experiments resulted in failure from lack of a suitable host, namely, man; rather than from lack of a suitable method of transmission.

BIBLIOGRAPHY

Bullock, F. D., and M. R. Curtis: Spontaneous Tumors of the Rat. *J. Cancer Research*, 14:1–115, 1930.

Cunningham, W. F., and K. McAlpin: Experiments with Hodgkin's Disease. An Attempt to Produce it in Anthropoids and other Monkeys. *Arch. Int. Med.*, 32:353–358, 1923.

Forbus, W. D., and C. L. Davis: A Chronic Granulomatous Disease of Swine with Striking Resemblance to Hodgkin's Disease. *Am. J Path.*, 22:35–49, 1946.

Gillman, J., and T. Gillman: Lymphomata (including Hodgkin's-like Sarcomata). Their Experimental Production, *Clin. Proc.*, 8:222–359, 1949.

Gye, W. E., A. M. Begg, Ida Mann, and J. Craigie: The Survival of Activity of Mouse Sarcoma Tissue after Freezing and Drying. *Brit J. Cancer*, 3:259–267, 1949.

Hoster, H. A., and M. B. Dratman: Hodgkin's Disease (Part I and II), *Cancer Research*, 8:1–78, 1948.

Longcope, W. T.: Notes on Experimental Inoculations of Monkeys with Glands from Cases of Hodgkin's Disease. *Bull. Ayer. Clin. Lab.* Pennsylvania Hosp., No. 4:18–25, 1907.

Reed, D. M.: On the Pathological Changes in Hodgkin's Disease, With Special Reference to its Relation to Tuberculosis, *Johns Hopkins Hosp. Rep.*, 10:133–196, 1902.

EFFECTS OF MAZE RUNNING AND SONIC STIMULATION ON VOLUNTARY ALCOHOL INTAKE OF ALBINO RATS*

WILLIAM T. MOORE, BERNICE M. MOORE, JOE B. NASH† AND G. A. EMERSON

There is a marked individual variation in consumption of alcohol among white rats given free choice of drinking ethanol in 5–10% V/V concentrations or water. Relative amounts of ethanol and water ingested under these conditions are usually quite uniform over considerable periods for each rat, but differences between rats may be very large. Such differences have been attributed to genetic and nutritional differences by Williams *et al.* (1947, 1950), and the rationale of massive vitamin therapy of chronic alcoholism has received support from this viewpoint (Williams, 1951).

Williams *et al.* (1950) note four types of behavior of rats in consumption of alcohol: some drink large amounts from the start, others avoid alcohol for a few weeks and then drink large amounts, still others avoid alcohol uniformly for many weeks, and a few are highly irregular in their choice of alcohol. With some 300 of the strain of rats used in the present work, the incidence of preference of alcohol over water averages about 8%, initially. Although some rats having a high initial alcohol intake may show periods of somewhat decreased consumption of alcohol, none in our experience has reverted to a low intake within a period of 160 days.

A difference in behavior of rats in regard to choice of alcohol or water suggests the possibility that other forms of behavior may vary in a parallel manner in individual rats (Moore *et al.*, 1951). If correlations of a form of behavior of unknown etiology were found with other forms of behavior in which relations to definite psychologic criteria are more or less accepted

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(Munn, 1950), some insight might be had into the mechanism of the former. Neither ability in learning, as measured by maze running behavior, nor excitability of the motor cortex, as measured by susceptibility to audiogenic shock, was found to be unequivocally related to preference for alcohol, although the numbers of rats studied which frankly showed a decided preference for alcohol were too small in each instance to permit final conclusions. It was noted, however, that some of the rats showed heightened preference for alcohol during the period in which they were subjected to the above unusual experiences. Analysis of this effect is made in the present study.

Experimental

Twenty healthy, adult white rats, weighing 150–200 g., were kept in individual cages with constant access to 5% alcohol and water. Positions of the two drinking-bottles were interchanged daily. This technique follows thatt of Williams *et al.*, (1950). Special drinking tubes, with cup-shaped ends to avoid spillage, were designed by Professor P. L. Ewing. All rats were given a surplus of dog chow throughout the experiments.

Maze Running.—The 20 rats were first trained to run a multiple-T maze having 12 correct turns between entrance and goal, and 38 incorrect turns into and including blind alleys. A 24-hour thirst was the motivation towards reaching a drinking-bottle at the goal. After becoming proficient, the rats were permitted a choice of 5% ethanol or water for 10 days, and then made to run the maze every other day for 14 days. During this time, they were given a choice of 5% ethanol or water on the days on which they were not deprived of fluid intake.

Sonic Stimulation, every other day.—The rats were next maintained under conditions where they had a choice of 5% ethanol or water, for 89 days. They were then given on alternate days an auditory stimulus, sufficient to cause audiogenic shock in some, for 12 days. The apparatus used for the auditory stimulation was designed to be fairly sound-proof, and consisted of a wooden box measuring 25½ x 20½ x 16¾ inches, with a thin sheet-metal box measuring 15 x 23 x 12 inches

within it; the tops of both were open, and cotton insulation separated the boxes on all other sides. The larger box was covered by a window made of two sheets of glass separated by an air-space, held in a wooden frame. Sound was produced by an electric door-bell placed at the top of the inner box. Each rat was subjected to sound for 2 minutes, during which time its motor activity was noted.

Sonic Stimulation, twice a day.—During a third control period of 31 days, the dats were permitted a choice of water or 10% ethanol. Following this, they were subjected to auditory stimulation twice a day by the method described above, for 14 days. One of the 20 rats died during this period.

Results

The present findings confirm the large variation between individual rats in their intake of ethanol under conditions of free choice, as noted by Williams *et al.* (1950). During the initial 10-day period, actual volumes of 5% ethanol consumed per day varied from 0–44.5 cc., equivalent to 0–12.73 cc/kg. of absolute alcohol. Preference for alcohol, expressed in % as the volume of 5% ethanol / the total volume of fluid consumed per day x 100, also showed a marked variation from 0–68.6% during this period. Williams *et al.* (1950) note variations in intake of absolute alcohol from 0.3 to 21.7 cc/kg/day for rats given special diets. Although extrapolations of the higher levels of intake to man, amounting to 3.7 qt. of 90 proof spirits according to Williams *et al.* (1950), would indicate a strongly narcotic dose, none of the rats in the present study showed any symptoms of depression. Until such techniques are developed as will produce objective evidence of moderate to severe alcoholic intoxication in rats permitted free choice of alcohol and water, valid conclusions may not be drawn directly as to effects on man of treatments affecting alcohol intake in rats.

In presenting and interpreting the present results, we prefer to avoid the term alcoholism in relation to levels of alcohol intake by rats, since there are no apparent physiological corollaries with alcoholism in man, even at the maximum rates of intake in rats. Conversely, we belive that preference for alcohol in rats can be measured accurately, and that relative

alcohol intakes are more significant than the absolute; results are therefore noted as % preference for alcohol, as defined above.

We have confirmed the statement of Williams *et al.* (1950) that at least some rats tend to increase, in time, their preference for alcohol. In the 1st control period of 10 days, an average of 20.95% of the total fluid intake was 5% ethanol; in the 2d control period of 89 days, 38.21% of the average total fluid intake were 5% ethanol; and in the 3d, of 21 days, 40.62% were 10% ethanol. Increases in alcohol preference during experimental periods subsequent to these control periods should be interpreted with this normal temporal increase in mind; if results during the experimental periods are compared with later control periods, none indicates any significant changes.

Maze Running.—During the 14-day period of maze running, the rats increased their preference for alcohol by 26.8%, from an average control level of 20.95% of 5% alcohol in their total fluid intake to 26.57%. This difference is not statistically significant. Of the 20 rats, 11 appreciably increased their alcohol preference, 4 decreased it and 5 showed almost no change.

Sonic Stimulation, every other day.—During the 12-day period in which the rats received auditory stimulation every other day, they increased their preference for alcohol by 18.1%, from an average control level of 38.21% of 5% alcohol in the total fluid intake to 45.13%. This difference is *statistically* significant, at the $p = 0.01$ level. Twelve of the 20 rats increased their preference for alcohol, 7 decreased it and 1 was unchanged.

Sonic Stimulation, twice a day.—During the 14-day period in which auditory stimulation was given twice a day, the rats decreased their preference for alcohol by 14.3%, from an average control level of 40.62% of 10% alcohol in the total daily fluid intake to a level of 34.22%. This is not statistically significant. Of the 19 rats, 11 decreased their preference for alcohol, 4 increased it, and 4 were virtually unchanged.

Discussion

It is tempting to interpret the marked increases in alcohol preference of some of the rats subjected to the first 2 experi-

mental procedures in anthropomorphic terms. If unusual experiences induce in rats a condition similar to anxiety in man, increased consumption of alcohol could acceptably be considered to reduce the anxiety by a correspondingly increased depressant action, thus liberating energy otherwise utilized to control the anxiety, and through this mechanism producing an effect beneficial to the rats which might so be recognized by them, leading to continued high alcohol intake. Failure to produce more significant increases in alcohol preference under the 1st 2 experimental conditions could easily be explained by inadequacy of the "stress." Similarly, in the 3d experimental condition, no real difficulties are met with in explaining a decrease in alcohol preference: adaptation, by frequent repetition of the "stress," would result in less anxiety, and therefore less need for a depressant to release energy bound by the anxiety; in point of fact, Humphrey and Marcuse (1940) have clearly noted that repetition of the stimulus, handling, and neuromuscular activity will decrease susceptibility to audiogenic convulsions in the rat.

A minor difficulty in this interpretation is the observation that no objective symptoms of depression occur in rats at any of the alcohol intakes noted in the present study. We doubt that it is possible that every rat, in its behavior regarding voluntary choice of alcohol over water, is comparable to the hypothetical human alcoholic who copes with his situation ideally with just an adequate amount of alcohol to release the maximum energy bound by his anxiety, without ever showing depression; we hold this belief despite the implication that rats in many ways are more rational organisms than is man (Tolman, 1945).

It is of interest that widely different degrees of statistical validity of the experimental results are obtainable when accepted methods of statistical analysis are applied to the magnitude of changes in absolute amounts of alcohol consumed, the average alcohol preferences, and the numbers of rats showing increases or decreases in preference for alcohol. It is recognized that the queen of sciences yields us the most accurate measures of the validity of each criterion studied; yet, it is difficult to reconcile the quantitatively and even qualitatively very large differences in the validity of the different criteria,

when all of these criteria superficially indicate the same meaning of the results. It would appear that the application of statistics to psychological problems, which application has had such tremendous vogue, might well enquire into other factors than the truthfulness of the data with which it deals.

Summary

Twenty albino rats were observed as to their choice of 5–10% V/V ethanol solutions over water, and were then subjected to the unusual experiences of re-learning a multiple-T maze, and sonic stimulation to an intensity producing audiogenic shock in some, given every other day or twice a day. Some rats significantly increase their preference for alcohol over water under these "stress" conditions. These results are discussed in regard to mechanisms of releasing energy otherwise utilized in controlling anxiety. No support is gained from the present study that alcoholism is primarily dependent on psychic rather than genetotrophic factors, and the following observations may support the importance of the latter: the marked variation in preference of individuals for ethanol solutions over water, and the increase with time in preference for alcohol. Conversely, normal distribution of criteria other than preference for alcohol might be construed to minimize genetic factors. Pitfalls in applications of statistical methods to complex psychologic problems are noted. Further study relating abnormal preference of rats for alcohol to other, interpretable forms of behavior of these relatively simple organisms is indicated.

References

Humphrey, G., and F. Marcuse: Factors involved in the immunity of rats to convulsions under intense auditory stimulation. Bull. Canad. Psychol. Assoc., Oct. 1–2, 1940; *v.* also, Factors influencing susceptibility of albino rats to convulsive attacks under intense auditory stimulation. *J. Comparative Psychol.*, 32:285–306, 1941.

Moore, W. T., B. M. Doucet, H. G. Tree, and P. L. Ewing: Psychic stress and voluntary choice of ethanol in rats. *Fed. Proc.*, 10:326, 1951.

Munn, N. L.: Handbook of Psychological Research on the Rat. An Introduction to Animal Psychology. pp. xxvi + 598. Houghton Mifflin Co., Cambridge, Mass., 1950.

Tolman, E. C.: A stimulus-expectancy need-cathexis psychology. *Science*, 101:160–166, 1945.

Williams, R. J.: The etiology of alcoholism: a working hypothesis involving the interplay of hereditary and environmental factors. *Quart. J. Stud. Alc.*, 7:567–587, 1947.

Williams, R. J., L. J. Berry, and E. Beerstecher: Genetotrophic diseases; alcoholism. *Texas Repts. Biol. and Med.*, 8:238–256, 1950.

Williams, R. J.: Nutrition and Alcoholism. University of Oklahoma Press, Norman, Oklahoma, 82 pp., 1951.

THE METABOLISM OF GLUCURONIC ACID IN MAN*

ANDREW A. ORMSBY

Glucuronic acid, usually considered only in its role as a structural carbohydrate and as a component of detoxication products, was once thought to be a normal intermediate in the oxidation of glucose. This view is found in many standard textbooks of the 1914 era. Thus in the third edition of Abderhalden's Lehrbuch der physiologischen Chemie (1914) we find the statement, "Wir kommen somit zum Schlusse, dass nichts dagegen spricht, dass die Glucuronsäure ein ganz normales Zwischenprodukt beim Abbau der Glucose ist." The experimental basis for this widely accepted conclusion is found in the work of P. Mayer (1902). Following the subcutaneous injection of 5 gm. of the sodium salt of glucuronic acid in man, Mayer could find no reducing substances in the urine. After the injection of larger amounts (10–19 gm.) the urine had strong reducing properties which Mayer showed, by the isolation of the osazone, to be glucose. Biberfeld (1914), on the other hand, injected glucuronic acid into dogs and rabbits and recovered the material quantitatively in the urine, concluding that glucuronic acid is not oxidized in these animals. In view of Biberfeld's work and the fact that it soon became apparent that glucuronic acid is not a normal intermediate in the oxidation of glucose, the prevailing opinion in recent years has been that glucuronic acid is not oxidized. Experimental evidence has been meagre for two reasons; it is only recently that glucuronic acid has become readily available for feeding experiments, and the methods of analysis for glucuronic acid in blood and urine have been very poor. The inadequacy of methods is reflected in the lack of agreement on normal levels in blood and urine in man (Table I).

There has been indirect evidence that glucuronic acid is oxidized in man. Enklewitz and Lasker (1935) found that the elimination of pentose in cases of pentosuria was greatly increased by the administration of glucuronic acid. The increase

*From the Department of Biochemistry and Nutrition, The University of Texas Medical Branch, Galveston. Received for publication October 10, 1951.

TABLE I

Normal Levels of Glucuronic Acid in Man

Blood mg. %	Urine mg./24 hrs.	Reference
—	65—306	Deichmann and Thomas
—	375—456	Maughan et al.
0.4—2.5	—	Deichmann and Dierker
6.8—9.2	—	Ratish and Bullowa
2.3—6.6	—	Laing and Anderson

was shown to be due to l-xyloketose. Flaschenträger, *et al.* (1945) found that the excretion of furan-2:5-dicarboxylic acid in man was increased 5 to 15 fold when 5 gm. of glucuronic acid was administered. It is difficult to evaluate this work quantitatively since furan-2:5-dicarboxylic acid is itself largely oxidized in the body. It was demonstrated that Escherichia coli do not form furan-2:5-dicarboxylic acid from glucuronic acid, therefore it is unlikely that the conversion was caused by bacteria in the gut.

The experiments to be described were designed to test further the ability of man to oxidize glucuronic acid. Five gram portions of glucurone (glucuronic acid lactone) were fed to normal adult males and the urine analyzed for glucuronic acid. Basal levels were established by repeated analyses without the administration of glucurone.

EXPERIMENTAL

Analysis of urine for glucuronic acid. Most procedures used in the past have been based on the reaction of glucuronic acid with naphthoresorcinol. This is a non-specific reaction and considerable difficulty has been experienced in controlling conditions so that reproducible results might be obtained. As indicated by the wide variation of normal values (Table I) the method has been highly unsuccessful.

The method of analysis used here employs the carbazole reaction of Dische (1947). The method was used essentially as described by Dische, except that a blank was run to take into account the color produced by the action of sulfuric acid on urine in the absence of carbazole. The blank was prepared as described by Dische except that 0.2 ml. of alcohol was added in

place of 0.2 ml. of an alcoholic solution of carbazole. Commercial carbazole gives a green color with the reagents but this is readily eliminated by using carbazole which is recrystallized twice from benzene. If the sulfuric acid contains suspended particles of charred material, common even in the best grades, it is necessary to remove these by filtration through glass wool. The method was found to be very sensitive, the usable range (Coleman Junior Spectrophotometer) being 5–70 gamma per sample.

The basal level of glucuronic acid excretion was obtained by the analysis of three or four 24 hour urine specimens before beginning the experimental period. The urines were refrigerated and preserved with toluene and chloroform. After a basal level had been established 5 gm. of glucurone* was given orally and the glucuronic acid excretion determined during the two succeeding 24 hour periods. All extra glucuronic acid was found to be eliminated during the first 24 hours following ingestion.

Results and Discussion

The results of the feeding experiments are shown in Table II.

The disappearance of 80–85% of the ingested glucuronic acid was not startling in view of the divergent results reported in the literature. The high daily excretions during the basal periods were, however, very surprising. The carbazole method is much more specific than methods based on the naphthoresorcinol reaction and it was expected that lower normal levels would be found.

In order to check the validity of the method the absorption spectrum of the color obtained with pure glucuronic acid was compared with that obtained from urine. Glucose and the pentoses react with carbazole but higher concentrations of these sugars are required to give a color and the absorption spectra do not resemble that obtained from glucuronic acid. Absorption spectra were run with the Beckman spectrophotometer, using 1 cm. cells. The urine curve was obtained by subtracting the absorption of a urine blank containing no carbazole. From the results (Fig. 1) it is evident that the substance giving the color in urine is actually glucuronic acid.

TABLE II

Subject	Glucurone Fed	Glucuronic Acid in Urine mg./24 hrs.	Average Basal Level mg./24 hrs.	Recovery mg.	Recovery per cent
AO	None	665			
	None	685			
	None	594			
	None	627			
	5 gm.	1748			
	None	662	647	1101	22.0
DV	None	614			
	None	581			
	None	570			
	5 gm.	1302			
	None	503	567	735	14.7
BD	None	531			
	None	557			
	None	568			
	None	586			
	5 gm.	1384			
	None	533	555	829	16.6
CA	None	548			
	None	576			
	None	451			
	5 gm.	1326			
	None	477	513	813	16.3

*Supplied through the courtesy of Commercial Solvents Corp., New York.

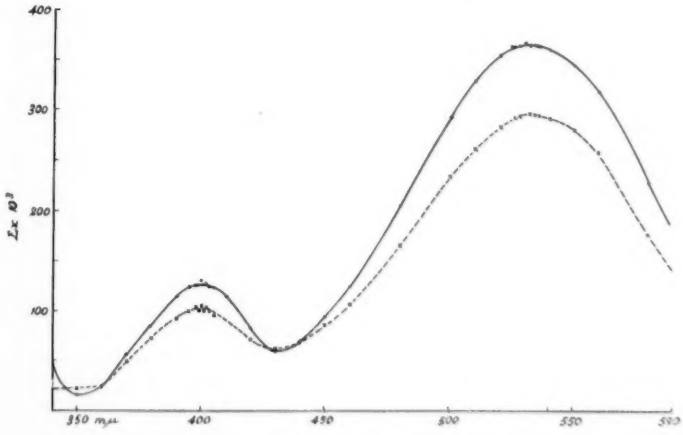

FIG. 1. Absorption spectra of colors obtained by the carbazole reaction applied to 50 gamma of glucuronic acid (solid line) and to 0.1 ml. of human urine (broken line).

A further check on the method was provided by the determination of recoveries of glucuronic acid added to urine (Table III).

TABLE III

Recovery of Glucuronic Acid Added to Urine

Glucuronic Acid Originally Present mg. %	Glucuronic Acid Added to 100 ml. Urine	Glucuronic Acid Found mg. %	Recovery	
			mg.	per cent
48.2	25	71.2	23.0	92
35.4	50	85.8	50.4	101
56.1	100	146.7	90.6	91
48.4	150	187.6	139.4	93

A complicating factor in all glucuronic acid analyses is that glucuronic acid exists in the urine in conjugated forms. Artz and Osman (1950) state that the free form is never found in nature, although this would be difficult to prove. In the naphthoresorcinol methods it is common practice to hydrolyze the conjugated glucuronic acid by refluxing with acid, although it has been shown that this may result in destruction of glucuronic acid. Using a typical procedure, we added 5 ml. of concentrated hydrochloric acid to 50 ml. of urine and refluxed for 30 minutes. Before hydrolysis the urine contained 48.2 mg.% glucuronic acid; after hydrolysis only 42.5 mg.% was found. Since it was evident that hydrolysis resulted in destruction, all of the values reported here were determined on unhydrolyzed urine. Glucuronic acid may be conjugated with a variety of substances (phenols, aromatic hydrocarbons and acids, sulfonamides, terpenes, sex hormones, etc.) and practically no data are available on the behavior of these conjugates with either naphthoresorcinal or with carbazole.

Many of these compounds are labile and isolation is very difficult. Hydrolysis may also occur due to the presence of beta-glucuronidase in urine. In most of the literature it appears that no preservative was used, or at least none is mentioned. Mercuric chloride has been reported to be of value in inhibiting glucuronidase, but the appearance of free menthol in rabbit urine containing menthol glucuronide and mercuric chloride makes us doubt its efficacy. Dische (1947) has tested his method on menthol glucuronide and claims that glucuronic acid in this

combination gives 15% less color than the corresponding amount of free glucuronic acid. We have prepared pure menthol glucuronide from the urine of rabbits which had been fed menthol, using the method of Quick (1924), and found no difference between the color given by free glucuronic acid and that in combination. Hence, for the present, it is assumed that conjugation does not affect the reaction of glucuronic acid with carbazole. Since the method involves heating the urine for 20 minutes in 72% sulfuric acid it is not unlikely that all of the above conjugates would be completely broken down, and that any effect on the color given by the glucuronic acid would be caused by interference by the products formed from the other member of the conjugate pair.

Since this work was finished a paper has appeared, by Fishman, *et al.* (1951), in which, using a modified naphthoresorcinol method, essentially the same results were obtained as those reported here. The emphasis in the investigations of Fishman, *et al.*, has been placed on the blood levels following feeding of glucuronic acid, and few results are given on urine. It is impossible, from the data presented, to calculate exact urine recoveries, but it is apparent that the recoveries, as well as the normal levels found, are on the same order as those presented here. The demonstration by Fishman of the high blood levels reached within a short time (30–60 minutes) following ingestion of glucuronic acid indicate that rapid absorption from the gut must take place. The rapid disappearance from the blood, with the appearance of only relatively small amounts in the urine, indicates rapid utilization. It is also of interest, in considering the fate of the glucuronic acid, that the blood glucose and the phosphate were found to be unaffected by feeding glucuronic acid.

Conclusions

When glucuronic acid is fed to man a large amount (80–85%) can not be recovered in the urine.

The basal urinary excretion of glucuronic acid appears to be on the order of 400 to 700 mg. per day in man.

BIBLIOGRAPHY

Artz, N. E., and Elizabeth M. Osman: Biochemistry of Glucuronic Acid, Academic Press, New York, 1950.

Biberfeld, J.: Zum Verhalten der Glucuronsäure im Organismus. *Biochem. Zeit.*, 65:479–496, 1914.

Deichmann, W. B., and G. Thomas: Glucuronic Acid in the Urine as a Measure of the Absorption of Certain Organic Compounds. *J. Ind. Hyg. Toxicol.*, 25:286, 1943.

Deichmann, W. B., and M. Dierker: The Spectrophotometric Estimation of Hexuronates in Plasma or Serum. *J. Biol. Chem.*, 163:753–760, 1946.

Dische, Z.: A New Specific Color Reaction of Hexuronic Acids. *J. Biol. Chem.*, 167:189–198, 1947.

Enklewitz, M., and M. Lasker: The Origin of l-Xyloketose (Urine Pentose). *J. Biol. Chem.*, 110:443–456, 1935.

Fishman, W. H., M. Smith, D. B. Thompson, C. D. Bonner, S. C. Kasdon, and F. Homburger: Investigation of Glucuronic Acid Metabolism in Human Subjects. *J. Clin. Invest.*, 30:685–696, 1951.

Flaschenträger, B., B. Cagianut, and F. Meier: Die Bildung von Furan-2, 5-Dicarbonsäure aus d-Glucuron und d-Galakturonsäure beim Menschen. Ein neuer Abbauweg für Kohlenhydrate. *Helv. Chim. Acta.*, 28:1489–1496, 1945.

Laing, G. H., and D. Anderson: Glucuronic Acid. Method of Determination and Normal Blood Levels. *Abstracts, Am. Chem. Soc., Division of Biol. Chem.*, Detroit, p. 610, 1950.

Maughan, G. B., K. A. Evelyn, and J. S. L. Browne: A Method for the Quantitative Estimation of Glucuronic Acid and Conjugated Glucuronides. *J. Biol. Chem.*, 126:567–572, 1938.

Mayer, P.: Eperimentelle Untersuchungen über Kohlenhydratsäuren. *Zeit. f. klin. Med.*, 47:68–108, 1902.

Quick, A. J.: A Method for the Quantitative Determination of Menthol Glycuronic Acid in Urine. *J. Biol. Chem.*, 61:667–677, 1924.

Ratish, H. D., and J. G. M. Bullowa: Determination of Glucuronic Acid in Biological Media. *Arch. Biochem.*, 2:381–388, 1943.

OBSERVATIONS ON A BARTONELLA ORGANISM IN CATTLE

M. Pollard and D. G. Parmer†

This is a brief report describing the detection of a Bartonella-like organism in the erythrocytes of local cattle *(Bos taurus)*.

During the third week of July, 1951, a local practicing veterinarian (D. G. P.) submitted citrated blood specimens from 2 mature Hereford cows, from a large ranch located 15 miles West of Galveston on Galveston Island. Twelve animals in a herd of 560 manifested rapid loss of weight, anemic mucous membranes, marked weakness, rapid shallow respirations and had rectal temperatures ranging from 103.7° F. to 104.6° F. One of the 2 whose blood was examined, collapsed following exertion, became cyanotic, and required physical resuscitation. This latter animal had lost approximately 350 pounds during the first week of illness. This illness was diagnosed clinically as anaplasmosis, except that neither hemoglobinuria nor icterus was observed. Subsequently, both animals died (one at 1 week after onset and the other at 3 weeks). Post-mortem examination of the first revealed a generalized anemic state, friable liver, and some patchy areas of pulmonary congestion. The histopathological examination of tissues was non-contributory due to post-mortem degeneration and inadequate fixation of the tissues.

The animals were heavily infested with horn flies *(Haematobia irritans)* and moderate numbers of hard ticks *(Amblyomma maculatum)**. Those were eradicated promptly: the former with DDT and the latter with arsenical dips.

Thin smears were prepared from the citrated blood, stained with Wright's then Giemsa stains and air dried. When examined with the oil immersion lens (1000X), about 20% of the erythrocytes contained organisms closely resembling those described by others (Weinman 1944) as Bartonella. The erythrocytes were distorted and showed evidence of anisocytosis

*We are indebted to Dr. Don W. Micks, The University of Texas, Galveston, for identification of these ectoparasites.

†Department of Preventive Medicine, The University of Texas, Medical Branch, Galveston.

and hypochromasia. In the central portions of the erythrocytes were basophilic pleomorphic organism (0.5–1.5 μ in length) ranging in appearance from coccoid, streptococcoid, to bacilliform shapes (Figure I). Such organisms were not observed in the white blood cells.

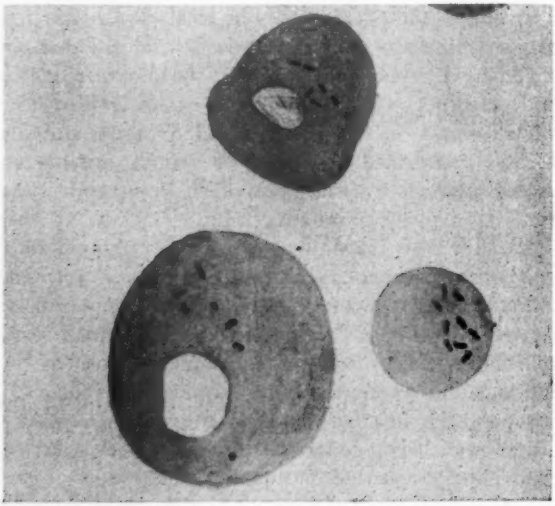

FIG. 1. Erythrocytes from blood of cow showing Bartonella organisms. Areas of rarefaction are lighter stained. Camera lucida drawing—about 2500 x. Wright-Giemsa stain.

Bovine Bartonellosis has been reported from Palestine by Adler and Ellenbogen (1934), from Algeria by Donatien and Listoquard (1934), and from Granada by Rodriguez (1935). From the available literature, such organisms as these have not yet been described in cattle of the Western Hemisphere. The organisms described by us may not have been responsible for the pathogenic effect observed and may have been coincident with another unrecognized affection; however, the primary anemia manifested by this illness, without intra-vascular hemolysis, suggests that the illness may conceivably be due to the organism observed in the blood. Bartonellosis may not actually

be a rare disease in this area, but may be so designated because of the infrequent examination of blood smears from cattle in this area.

REFERENCES

Adler, S., and V. Ellenbogen: A note on two new blood parasites of cattle. *J. Comp. Path. and Therap.*, 47:219, 1934.

Donatien, A., and F. Lestoquard: Sur une bartonella nouvelle du boeuf. Bartonella bovis n. sp. *Bull. Soc. Path. Exat.*, 27:652, 1935.

Rodriguez, I. G.: Inclusiones en los hematies de un bovido identificados con parasito del grupo Bartonella. *Rev. del Instituto Llorente*, 13:5, 1935. (Abstract in *Bull. Inst. Pasteur*, 34:1033, 1936.)

Weinman, David: Infectious Anemias due to Bartonella and related red cell parasites. Trans. of the American Philosophical Society. N. 5, 33: part III, 1944.

A CONSIDERATION OF SOME OF THE THEORIES RELATIVE TO THE ETIOLOGY AND INCIDENCE OF LUNG CANCER*

R. H. Rigdon and Helen Kirchoff

During the past fifty years it has been suggested by many that cancer of the lung is increasing. However, today, there is insufficient evidence to prove that this increase is either apparent or actual. Data showing the frequency of cancer are compiled in this country primarily from death certificates. These records give an adequate sample for statistical study; however, the authenticity of many of these death certificates may be questioned. The United States Public Health Service (1950) has observed from their study of death certificates that the diagnosis of cancer is more reliable than the site of origin of the neoplasm.

Improved methods in diagnoses have contributed much to an improvement in our statistics on cancer. However, the data given today on the frequency of cancer, when compared with that of former years, results in a "questionable state of knowledge" relative to the basic problem of the actual or apparent increase in the frequency of cancer of the lung. Dr. H. G. Wells (1927) discussed this problem approximately twenty-five years ago and indicated then the state of confusion relative to vital statistics. He said, "It seems not unfair to state at this time that there are few, if any, facts concerning cancer that can be established beyond dispute by the published statistical data . . . No matter what the question may be, the statistics that have been published will be found to give widely varying answers leading to all possible conclusions." The ratio of error in diagnosis, according to Wells (1927), may be as much as 50 per cent.

The advice given in 1929 by Weller relative to the diagnoses of cancer of the lung certainly is as true today as it was twenty years ago. He said, "Only those reputed examples of primary carcinoma of the lung are of value on which a complete autopsy

*From the Department of Pathology, The University of Texas Medical Branch, Galveston. Received for publication September 19, 1951.

has been done . . . There must be microscopic verification of each case, if the results are to be fully accepted . . . Every experienced prosector can recall autopsies in which painstaking search failed to disclose a primary site of malignant growth although abundant secondaries were present in the lungs or the liver . . . . Cases are encountered too, in regard to which positive conclusions cannot be drawn even with thorough microscopic study . . . . In studies which are only statistical, the reader is at a loss to know how critical the compiler may have been in selecting his original units."

Cancer is a disease primarily of adults. Cancer of the lung usually occurs after forty years of age. There has been a progressive increase in the number of individuals reaching the age of forty-five to sixty-five years since 1850 (Dorn, 1950). Today, this number is more than double what it was one hundred years ago (Table I). This factor alone will contribute to the

TABLE I

*Percent Distribution by Age Total Population—United States**

Year	Age 0–19	20–44	45–64	65+	Total
1850.....	52	35	10	3	100
1860.....	51	36	10	3	100
1870.....	50	35	12	3	100
1880.....	48	36	13	3	100
1890.....	46	37	13	4	100
1900.....	44	38	14	4	100
1910.....	42	39	15	4	100
1920.....	41	38	16	5	100
1930.....	39	38	18	5	100
1940.....	34	39	20	7	100
1950.....	33	38	21	8	100

*Data obtained from U.S. Bureau of Census.

increase in the number of cases of cancer of the lung that one sees today. In 1912, Adler wrote, "The physician must be imbued with the conviction that malignant pulmonary disease occurs much more frequently than is commonly believed." Frissell and Knox (1937) have called attention to the fact that many tumors of the lung, classified as sarcomata by pathologists of the last century, are now included as epithelial tumors. Formerly, when carcinomata of the lung were found they were

usually considered metastatic, since Virchow had stated that organs in which epithelial tumors metastasized were rarely the seat of primary carcinomata. Interest in the problem of cancer of the lung had led to the discovery of a considerable number of small pulmonary neoplasms with large metastases. Such metastases earlier observers undoubtedly regarded as the primary lesion (Frissell and associates, 1937). Steiner, *et al.* (1950) think that the "factor concerning pathologists" relative to the increase frequency of cancer of the lung "deserves to be dropped from further serious consideration. It has unjustifiably confused the problem too long.

Table II shows the number of deaths from cancer of the lung and the rate for each state as given by Vital Statistics for 1948 (Cancer Mortality in the United States, 1950). The smallest percentage, 2.9, is for Idaho, while the highest is 11.9 for New York. A variation then of 9 per cent occurs throughout the United States. This variation is not related to geographical areas except for the fact that Connecticut, Massachusetts and New York have the highest rate, varying from 10.2 to 11.9. The ratio in Alabama, the District of Columbia, Arizona and West Virginia varies between 5.0 and 5.4 per cent. This variation in distribution of cancer of the lung must be considered when the etiology of this type of neoplasm is considered.

In 1926 Green observed that the cancer death rates varied directly with the number of physicians per 100,000 of population. This variation was attributed to the fact that places where the rates are highest have better medical attention, hospitalization is common and post mortems are frequently made. Table II gives the number of practicing physicians in the different states in 1949 and also the number of persons per physician. The highest ratio of population to physician is in the District of Columbia where the death rate for cancer of the lung is 5.2 per cent. New York is second and the death rate for cancer of the lung is 11.9 per cent. Maine has a ratio of population to physician of 1,148 to one, and Wisconsin 1,134 to one, while the death rate of cancer of the lung is 5.8 and 6.4 respectively. Montant has a ratio of 1,098 to one and Louisiana 1,313 to one, while the death rate for cancer of the lung is 8.8 and 8.5, respectively. The disproportion between the ratio of population

TABLE II

Deaths and Death Rates for Cancer of the Lung, Physicians and Persons per Physician Ratio, and Hospital Beds and Persons per Bed Ratio in the United States and each State.

	Total Deaths Ca. of Lung*	Rate*	Physicians†	Persons per Physician†	Beds‡	Persons per Bed§
United States	10,493	7.2	150,417	981	413,875	366
Alabama	148	5.1	1,782	1,675	4,755	643
Arizona	38	5.4	652	1,092	2,192	342
Arkansas	67	3.5	1,372	1,345	2,966	643
California	646	6.2	12,719	793	22,055	479
Colorado	79	6.6	1,613	752	6,185	214
Connecticut	221	11.1	2,451	823	8,780	228
Delaware	19	6.3	320	1,006	1,269	250
Dist. of Columbia	45	5.2	1,526	511	3,210	249
Florida	181	7.4	2,203	1,197	5,354	517
Georgia	132	4.2	2,365	1,395	4,851	710
Idaho	17	2.9	436	1,323	1,437	409
Illinois	685	8.2	10,262	845	28,711	303
Indiana	244	6.2	3,543	1,115	7,639	515
Iowa	149	5.7	2,328	1,097	7,465	351
Kansas	123	6.5	1,606	1,163	5,382	354
Kentucky	129	4.5	2,152	1,316	5,909	498
Louisiana	219	8.5	2,005	1,313	5,064	529
Maine	52	5.8	790	1,148	2,835	322
Maryland	180	8.4	2,232	1,042	7,298	321
Massachusetts ..	475	10.2	6,311	767	15,986	293
Michigan	439	7.1	5,343	1,168	15,413	413
Minnesota	180	6.1	2,758	1,064	10,096	295
Mississippi	78	3.7	1,240	1,668	3,215	677
Missouri	367	9.4	3,815	1,029	11,380	347
Montana	45	8.8	512	1,098	2,825	209
Nebraska	103	8.0	1,313	970	4,509	293
Nevada	10	6.1	168	940	217	737
New Hampshire	53	10.1	605	874	2,058	259
New Jersey	464	9.7	5,557	864	13,745	351
New Mexico ...	17	3.0	407	1,555	1,500	454
New York	1,692	11.9	23,684	620	52,984	279
North Carolina..	151	4.0	2,499	1,548	9,484	428
North Dakota ..	24	4.1	411	1,423	2,554	242
Ohio	573	7.3	7,677	1,037	20,027	396
Oklahoma	130	5.7	1,826	1,170	4,144	538
Oregon	72	4.4	1,388	1,041	4,557	333
Pennsylvania ..	876	8.4	11,087	920	38,386	273
Rhode Island ...	55	7.4	805	981	2,355	336
South Carolina..	73	3.7	1,202	1,658	2,941	719
South Dakota...	28	4.6	441	1,410	2,616	249
Tennessee	129	4.1	2,362	1,378	6,050	544
Texas	428	5.8	6,427	1,178	17,325	445
Utah	30	4.5	623	1,090	1,707	403
Vermont	25	6.9	434	855	2,243	168
Virginia	181	5.9	2,302	1,413	7,084	468
Washington	103	4.2	2,171	1,081	7,250	328
W. Virginia ...	96	5.0	1,492	1,295	5,655	354
Wisconsin	211	6.4	2,983	1,134	11,833	290
Wyoming	11	3.9	217	1,781	379	766

*Deaths and death rates for cancer of the lung in 1948. Public Health Service, National Office of Vital Statistics, Dec. 7, 1950 (Personal communication).

†Active physicians in private practice and physicians-population ratio for 1949. Ratio based on unofficial provisional intercensus estimates of civilian population. From Population Division, Bureau of the Census, which takes account of preliminary 1950 census. Source: 1950 American Medical Directory, Table 3.

‡Table 1. Hospital facilities by states and by control. J.A.M.A., 143:30, 1950.

§Based on the 1950 census of population. U.S. Dept. of Commerce, Bureau of the Census. Series Pc-9 No. 1.

to physician and the rate of death from cancer of the lung is interesting. There appears to be a larger number of cases reported in Louisiana than in many of the other states.

The number of nongovernmental hospital beds in each state for 1949 and the ratio of population to these beds is also shown in Table II. In many of the states where the ratio of population to beds is low, such as South Carolina and Wyoming, the death rate from cancer of the lung is low. In contrast, when the ratio of population to beds is high, as in New York, Connecticut and Massachusetts, the rate of death from cancer of the lung is high.

Calculations based on the data in Table II show that the reported death rate for cancer of the lung is, on the average, higher in those states which have a large number of physicians relative to the size of the population than it is in states which have a relatively smaller number of physicians. Likewise, the same death rate is higher in states which have a large number of hospital beds in proportion to the population than it is in states with a relatively low number of beds. In states which have on the average one physician per 1,600 persons, this death rate averages about 4 per 100,000 and increases uniformly to 8 per 100,000 in states which average one physician per 800 persons. In states that average one bed per 750 persons the rate averages about 4 per 100,000 and increases uniformly to 7 per 100,000 in states averaging 250 persons per bed.

The above comparisons were made by Dr. J. Allen Scott, Statistician at the Medical Branch, and were based on the following correlation coefficients: $r = -.661$ between the death rate and persons per physician. (P from the Z transformation $= <.00001$); and $r = -.433$ between the death rate and persons per bed ($P = <.002$). The regression of the cancer rate on the persons per physician is expressed by the equation $Y = 12 - .005X$ and the regression of the death rate on persons per bed by $Y = 8.7 - .006X$.

Through the control of many infectious diseases lives have been saved that ultimately succumb to cancer. It has been observed that a high crude cancer death rate is evidence of a good state of public health, being high in direct proportion to the degree of control of preventable diseases (Wells, 1927). Twenty-

five years ago it was predicted that "should a means be found to cure or prevent the acute respiratory infections, there would result a marked augmentation of the cancer mortality in the following years" (Wells, 1927). Antibiotics and chemotherapeutic agents now have reduced the death rates from pulmonary infections and the frequency of cancer has increased.

Today, attention of both the physician and the public is centered upon the chest. The recognition of cancer of the lung has been aided greatly by recent surveys and new diagnostic procedures. The Metropolitan Life Insurance Company (Statistical Bulletin, 1950) has pointed out the fact that "physicians have now access in increasing measure to improved facilities and diagnostic aids, such as the X-ray, the bronchoscope and laboratory examinations of lung secretions. Then, too, the greatly widened use of the mass X-ray in the campaign against tuberculosis has led to the incidental discovery of many respiratory cancers. In addition, the organized campaign against cancer has made both the public and the medical profession more alert to the danger signals. All these developments have contributed to the discovery of an increasing number of cases of the disease and hence to its more frequent ceritification as a cause of death." Arkin and Wagner (1936) stated that twenty years ago only 5 per cent of primary cancer of the lung was diagnosed clinically, while today about 50 per cent is recognized during life.

A second factor that is difficult to evaluate in determining the frequency of cancer of the lung is heredity. In recent years it has been necessary to know the frequency of cancer in any strain of mice in which carcinogenic agents are given. Even in experiments where the virus of milk cancer is being studied, it has been suggested that mice be used that have a genetic susceptibility to the development of breast cancer (Barnum and Huseby, 1950). In human cases of malignancy, emphasis is now placed on the "cancer history." In some cases this history is important in determining the type of treatment to be used. The variation in frequency of cancer in different European countries may be affected by heredity. Brines and Kenning (1937) suggested this possibility in a study made in

Detroit. They found a wide variation in the frequency of cancer of the lung in people of different European extraction. Cancer of the lung is less frequent in the Negro than in white persons (Rigdon and Kirchoff, 1951), (Ochsner and DeBakey, 1947), (Steiner, 1944). Mexican women exhibit a high incidence of pulmonary carcinoma (Steiner *et al.* 1950).

In a recent study of autopsy cases, it was found that cancer of the lung occurred in 2.25 per cent of 2,124 white persons and 0.7 per cent of 1,566 Negroes (Rigdon and Kirchoff, 1951). This is suggestive evidence of a variation in the two races. Investigators have suggested that heredity may be a significant factor in the frequency of cancer of the lung in the Schneeberg miners of Central Europe. For three hundred years these mines have been worked by the same people. As is frequently the case in Europe, the occupation of the father is followed by the son. The miners live in isolated places and inbreeding often occurs. All these factors, according to Lorenz (1944–45), may lead to a hereditary susceptibility.

Much has been written on the relation of chronic pulmonary irritants to cancer of the lung. In considering this, it may be worthwhile to review the effect of chronic pulmonary inflam mation produced by specific bacterial agents. Pulmonary cancer at one time was considered not to occur with tuberculosis; however, cases of the two lesions have been observed in the same lung. This association gave support to the opinion that the cancer was secondary to the tuberculosis (Moses, 1925) (Wolff, 1895) (Ewing, 1928). Many studen's now consider this association as incidental and not one of cause and effect (Sison and Monserrat, 1927) (Koletsky, 1938). Hueper (1942) says, "Tuberculous infection of the lung has no causative relation to the majority of pulmonary malignancies, and does not play any role in the production of the recently observed increase of this tumor variety."

The influenza epidemic of 1918 and 1919 has been considered to be an etiological factor in the increase of cancer of the lung Winternitz, *et al.* (1920). Hueper (1942) pointed out that an increase of lung cancer did not follow the great influenza epidemic of 1888 and 1889 and, furthermore, the present increase started many years before the epidemic of 1918. Kerley (1938)

and Dungal (1950) have found that the inhabitants of Iceland are relatively free of lung cancer in spite of the fact that the last influenza epidemic was severe. Macklin and Macklin (1940) concluded from their study that influenza has not been proven as a cause of carcinoma of the lung.

Many chemical substances and physical agents have been suggested as carcinogens and as the etiology of cancer of the lung. These are discussed in an excellent publication by Hueper (1942). The many contradictory opinions, however, reflect the uncertainty of many of these agents as being carcinogenic. Kennaway and Kennaway (1947) concluded that industrial hazards did not play any important role in the increase of cancer of the lung. Silicosis has been suggested as either a direct or indirect cause of pulmonary cancer. According to Hueper (1942), "Silicosis does not predispose or favor the development of a pulmonary malignancy . . . There do not exist any experimental observations which support the existence of causal interrelation between the inhalation of silica dust and lung cancer." Pollution of the air by soot, tarry substances from the dust coming from tarred or oiled roads, and the combustion of distillation and fractionation products of tars and oils have been suggested as the cause of the increased frequency of cancer of the lung. Such data usually are unconvincing and the experimental results vary. If most lung cancer is caused by atmospheric carcinogens, widespread dispersion and nearly equal exposure within a given area would be expected, again excepting the special industrial groups (Steiner, *et al.*, 1950).

Relative to the increase frequency of cancer of the lung in Los Angeles County and the "smog," Steiner and associates (1950) say, "Neither the high incidence nor the increase is accounted for by the current climatic smog situation, since it is of recent advent, except that previous data on the latent period for induction of lung cancer may not be applicable. Neither, however, is smog exonerated, it should be judged as carcinogen or noncarcinogen on its own merits, as may be revealed by future investigations." Radioactive substances long have been considered as one of the etiologic agents for cancer of the lung. These substances, if proven to be carcinogenic, certainly will

not account for the increase in the number of cases of bronchogenic cancer now occurring.

The theory today that is more frequently discussed is "cigarette smoking." This, however, is not new. In 1898, Rottmann suggested that workers in a cigar factory developed cancer of the lung more frequently than others. Lehmann (1942) concluded that the increase in these workers was more likely the result of their smoking than their occupation. Arkin and Wagner (1936) suggested that tobacco smoke produced chronic irritation with epithelial metaplasia in the bronchi and bronchioles. Roffo (1939) thought that tar in the cigarette was responsible for cancer of the lung. McNally (1932) suggested that nicotine was the specific irritant. Daff and Kennaway (1950) said, "The only carcinogenic substance which has been found in tobacco smoke is arsenic." The evidence that arsenic can produce carcinoma of the lung is suggestive rather than conclusive (Hill and Fanning, 1948). Macklin and Macklin (1940) concluded that tobacco smoke has not been proven as a cause of cancer of the lung. A public Health Bulletin (Cancer Mortality in the United States, 1950) states, "There is no conclusive evidence that smoking is an important etiological factor" in cancer of the lung.

The frequency of cigarette smoking in patients with cancer of the lung has been given in the papers by Ochsner and DeBakey (1941), Wynder and Graham (1950), Schrek and associates (1950), and Doll and Hill (1950). Wynder and Graham (1950) found among 605 patients with cancer of the lung and 780 male hospital patients with diseases other than cancer of the lung that there were 98.7 per cent smokers in the cancer of the lung group and 85.4 per cent smokers in the non-cancer of the lung group. In a study by Doll and Hill (1950), of 649 males with cancer of the lung, there were 647 smokers, a percentage of 99.7. In the control group of 649 patients with diseases other than cancer of the lung, there were 622 smokers, a percentage of 95.8. Certainly, 99.7 and 95.8 per cent is not an impressive difference. It is of interest to observe also in the statistical study made by Wynder and Graham (1950) that "the greater practice of inhalation among cigarette smokers is believed to be a factor in the increased incidence of the disease."

Doll and Hill (1950), in a similar statistical study, state that "it would appear that lung carcinoma patients inhale slightly less often than other patients." In a third statistical study, Schrek, *et al.* (1940) conclude, "This relative low percentage of deaths by cancer of the respiratory tract as compared to the high percentage of smokers indicates that smoking is, at most, only a weak carcinogenic agent."

Apparently there has been little data collected to show the frequency of smoking in the general population. Fortune Magazine (1935) conducted a survey and found that 37 per cent of the persons interviewed smoked cigarettes, 65.5 per cent of the men under 40 and 39.7 per cent over 40 years of age smoked, and 26.2 per cent of the women under 40 and 9.3 per cent over 40 years of age smoked. In November, 1950, Collier's Magazine (Maisel) estimated that "more than three-fourths of all adult men and more than 40 per cent of all women smoke." Of 310 white women, 20 to 29 years of age, interviewed in Columbus, Ohio, in 1947, 39.68 per cent smoked cigarettes (Mills and Porter, 1950). Of 520 white women of similar age, interviewed in Cincinnati, Ohio, in 1947, 41.35 per cent smoked cigarettes (Mills and Porter, 1950). Of 1,153 white men, 20 years of age and older, interviewed in 1947 by Mills and Porter (1950), 41.7 per cent smoked cigarettes, 25.6 per cent smoked a pipe, cigars or a combination of these, while 32.6 per cent were nonsmokers. The variation is great in the number of smokers in hospital patients, with and without cancer of the lung, when compared with those in the general population, as shown in the above surveys.

In 1950, Doll and Hill said that "over the last twenty-five years the deaths attributed to cancer of the lung have been much greater than the increase in tobacco consumption" in England and Wales. The increase in percentage of deaths attributed to cancer of the lung during the past thirty years in this country is also greater than the increased consumption per capita of tax-paid cigarettes. There has been a progressive increase in the per capita consumtion of tax-paid tobacco products in the United States since 1900 (Table III). An attempt has been made to obtain data relative to the number of tax-paid cigarettes sold in each state during the past twenty years

TABLE III

Per Capita Consumption of Tax-Paid Tobacco Products in the U.S.

Calendar Year	Cigarettes Number	Cigars Number	Smoking Tobacco—Lbs.
1900.....	32	73	1.07
1910.....	94	77	1.76
1920.....	419	80	1.36
1930.....	972	50	1.32
1940.....	1369	42	1.54
1950*.....	2384	37	.72

*Estimate by Bureau of Agricultural Economics, U.S.D.A.

in order to correlate these data with the frequency of cancer of the lung. Many states, however, have been taxing cigarettes for only a few years and, furthermore, it has been pointed out to us by the Comptroller of Public Accounts for the State of Texas (Calvert, 1951) that large quantities of tax-exempt cigarettes are consumed by the Armed Forces and until recently some cigarettes were shipped from one state into another for consumption. Therefore, little significance may be attached to data relative to tax-paid cigarettes consumed in each state.

Table IV, however, shows twenty-three states from the forty-one consulted in which satisfactory data were obtained on the number of tax-paid cigarettes sold since this specific tax was instituted. Only five years are used here to show the per capita number of cigarettes sold. The population for each state was obtained from the United States Vital Statistics. The death rate for cancer of the lung is shown only for 1948. Since there is apparently no relation between the average consumption of cigarettes as indicated by those taxed and the death rate for cancer of the lung in 1948 in these twenty-three states, it is considered unnecessary to include similar data for the years 1930, 1935, 1940 and 1945. The lung cancer death rate for Mississippi in 1948 was 3.7 and 311 tax-paid cigarettes were sold per person. In New Hampshire the lung cancer death rate was 10.1 and 3,053 tax-paid cigarettes were sold per person. The lung cancer death rate tripled, while the sale of tax-paid cigarettes per person increased 9.8 times. In Idaho, 2,003 tax-paid cigarettes were sold per person, while the lung cancer death rate was only 2.9; but in New York there were 2,319 tax-paid cigarettes sold per person and the lung cancer death

TABLE IV

*Per Capita Consumption of Tax-Paid Cigarettes**

States	Years 1930	1935	1940	1945	1948	Lung Cancer Death Rate 1948
Alabama	254	401	631	980	1,435	5.1
Arkansas				605	1,134	3.5
Connecticut ...			1,885	1,823	2,469	11.1
Florida				2,205	2,700	7.4
Idaho				1,176	2,003	2.9
Illinois				1,715	2,303	8.2
Indiana					2,352	6.2
Iowa				1,167	1,920	5.7
Kentucky			968	1,022	1,902	4.5
Maine				2,123	2,562	5.8
Minnesota					1,985	6.1
Mississippi		66	124	201	311	3.7
Montana					2,453	8.8
Nebraska					2,047	8.0
New Hampshire					3,053	10.1
New York			1,102	1,830	2,319	11.9
Ohio		1,081	1,509	2,373	2,383	7.3
Oklahoma				861	1,405	5.7
Pennsylvania ..				1,325	1,747	8.4
Rhode Island ..			1,734	1,739	2,607	7.4
Tennessee				1,005	1,705	4.1
Washington ...			1,263	1,496	2,107	4.2
Wisconsin					2,050	6.4

*Data obtained in personal communication from each state.

rate was 11.9. The cancer death rate, therefore, increased 4 times but the number of cigarettes sold per person was approximately the same in New York and Idaho. It is obvious from the data given in Table IV that there is no correlation between the number of cigarettes consumed per capita as indicated by tax-paid cigarette income and the number of deaths from primary cancer of the lung in the different states.

The variation in the frequency of cancer of the lung in the male and female has been attributed to the fact that females have been smoking for too short a period. If this is true, it would seem to us to be difficult to arrive at such a conclusion from the available data. Wynder and Graham (1950) found the frequency of cancer of the lung to be 18.5 to 1 in the male and female, while Ochsner and DeBakey (1941) found it to be 3.74 to 1. Lindskog (1948) found the ratio to be 4.5 to 1 from 1938 to 1943. The same investigator with Bloomer (1948) found it to be 24 to 1 in 1947 and 1948. This increase of male

smokers relative to female smokers from 4.5 to 24 within a period of four years is interesting.

As far as we know, Wynder and Graham (1950) are the only investigators who have separated the histologic types of bronchogenic carcinoma and attributed the smoking of cigarettes to be an etiologic factor for the one and not for the other. This will be a most interesting observation if it is confirmed by others and can be produced experimentally. Until more is known relative to all malignancies, care must be shown in considering one etiologic agent responsible for one type of cancer and something different for another type of malignant growth arising in the same anatomic area as the bronchi and the adjacent tissue.

Summary

There is such a wide variation in the significance of the theories advanced to account for the increase in the frequency of cancer of the lung that the data, in our opinion, do not justify the conclusion that cancer of the lung has actually increased as a result of any one so far advanced. The statistical data presented by some investigators relative to cigarette smoking are impressive; however, there is such a wide variation in control samples that, in our opinion, additional sampling is needed to warrant their conclusions. There is no significant correlation between the amount of tobacco consumed and the death rate for cancer of the lung in the United States. There is, however, a significant correlation in the United States between the population to physician ratio and the population to bed ratio and the number of reported deaths from cancer of the lung. Better diagnoses, decrease in the number of deaths from infectious diseases by both prevention and therapy, and the increase aging of the population seem to be more significant factors than any one or group of carcinogens to account for the apparent increase in the frequency of carcinoma of the lung.

We wish to acknowledge the assistance given to us in this study by the National Tobacco Tax Association. The states contacted were those with State Tobacco Tax Administrators. This information was given us by Mr. Charles F. Conlon, Executive Secretary of the National Tobacco Tax Association, 1313 East 60th St., Chicago, Ill.

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REFERENCES

Adler, I.: Primary Malignant Growths of the Lungs and Bronchi. Longmans, Green and Co., New York, 1912.

Arkin, Aaron, and David H. Wagner: Primary Carcinoma of the Lung. A Diagnostic Study of One Hundred and Thirty-Five Cases in Four Years. *J.A.M.A.*, 106:587–591, 1936.

Barnum, Cyrus P., and Robt. A. Huseby: The Clinical and Physical Characteristics of Preparations Containing the Milk Agent Virus: A Review. *Cancer Research*, 10: #9, 523–529, 1950.

Brines, Osborne Allen, and John Carl Kenning: Bronchiogenic Carcinoma. *Am. J. Clin. Path.*, 7:120–133, 1937.

Calvert, Robert S.: Personal communication, Jan. 23, 1951.

Cancer Mortality in the United States. Vital Statistics Special Reports, 32: #3, 43, July 19, 1950, U. S. Public Health Service.

Cancer Mortality in the United States. Vital Statistics Special Reports, 32: #4, 167, July 19, 1950. U. S. Public Health Service.

Cancer Mortality in the United States. Vital Statistics Special Reports, 32: #3, 45, July 19, 1950. U. S. Public Health Service.

Daff, M. E., and E. L. Kennaway: The Arsenic Content of Tobacco and of Tobacco Smoke. *Brit. J. Cancer*, 4: #2, 173–182, 1950.

Doll, Richard, and A. Bradford Hill: Smoking and Carcinoma of the Lung. *Brit. Med. J.*, #4682, 739–748, Sept. 30, 1950.

Dorn, Harold F.: Personal communication, Sept. 29, 1950. Source, U. S. Bureau of Census.

Dungal, Neils: Lung Carcinoma in Iceland. *Lancet*, 2:245–247, 1950.

Ewing, James B.: Neoplastic Diseases. A Treatise on Tumors. W. B. Saunders Co., Philadelphia, 1928, 3rd Edition, p. 852.

Fortune Magazine: The Fortune Survey—Cigarettes. Vol. 12, #1, 28, July, 1935.

Frissell, Lewis Fox, and Leila Charlton Knox: Primary Carcinoma of the Lung. *Am. J. Cancer*, 30:219–288, 1937.

Green, Howard Whipple: Cancer Mortality. *Am. J. Public Health*, 16: 605–609, 1926.

Hill, Bradford A., and E. Lewis Fanning: Studies in the Incidence of Cancer in a Factory Handling Inorganic Compounds of Arsenic. *Brit. J. Industrial Med.*, 5:1–15, 1948.

Hueper, W. C.: Occupational tumors and Allied Diseases. Charles C. Thomas, Springfield, 1942.

Kennaway, E. L., and N. M. Kennaway: Further Study of Incidence of Cancer of Lung and Larynx. *Brit. J. Cancer*, 1:260–298, 1947.

Kerley. (Reference from Matz, Philip B.: The Incidence of Primary Bronchiogenic Carcinoma. *J.A.M.A.*, 111:2086–2092, 1938).

Koletsky, Simon: Primary Carcinoma of the Lung. A Clinical and Pathologic Study of One Hundred Cases. *Arch. Int. Med.*, 62:636–651, 1938.

Lehamn. (Reference from Hueper—p. 394).

Lindskog, G. E.: Cancer of Lung. *Conn. Med. J.*, 12:1091–1095, 1948.

Lindskog, G. E., and W. E. Bloomer: Bronchogenic Carcinoma. Comparison of Two Consecutive Series of One Hundred Cases Each. *Cancer*, 1:234–237, 1948.

Lorenz, Egon: Radioactivity and Lung Cancer: A Critical Review of Lung Cancer in the Miners of Schneeberg and Joachimsthal. *J. Natl. Cancer Institute*, 5:1–15, 1944–45.

Macklin, M. T., and C. C. Macklin: Does Chronic Irritation Cause Primary Carcinoma of the Human Lung? *Arch. Path.*, 30:924–955, 1940.

Maisel, Albert G.: Don't Smoke—Unless You Like It. *Collier's Magazine*, Nov. 4, 1950, p. 8.

McNally, W. D.: The Tar in Cigarette Smoke and Its Possible Effects. *Am. J. Cancer*, 16:1502–14, 1932.

Mills, Clarence A., and Majorie Mills Porter: Tobacco Smoking Habits and Cancer of the Mouth and Respiratory System. *Cancer Research*, 10:539–542, 1950.

Moses, H. M.: Primary Carcinoma of the Lung. *Am. J. Med. Sc.*, 170: 102–118, 1925.

Ochsner, Alton and Michael DeBakey: Carcinoma of the Lung. *Arch. Surg.*, 42:209, 1941.

Ochsner, Alton, and Michael DeBakey: Primary Carcinoma of the Lung. *J.A.M.A.*, 135:321, 1947.

Rigdon, R. H., and Helen Kirchoff: Frequency of Cancer of the Lung in All Malignancies Studied at Autopsy. *Southern Med. J.*, 44:506–512, 1951.

Roffo, A. H.: Krebserzeugendes Benzpyren, Gewonnen aus Tabakteer. *Ztschr. f. Krebsforsch.*, 49:588–97, 1939. (Reference from Schrek et al).

Rottmann: Lung Cancers in Metal Workers, Cigar Workers and Spinners. *Inaug. Diss.*, Wurzburg, 1898. (Reference from Hueper).

Schredk, Robt., Lyle A. Baker, G. P. Ballard, and Sidney Dolgoff: Tobacco Smoking as an Etiologic Factor in Disease. *Cancer Research*, 10:49–58, 1950.

Sison, A. G., and C. Monserrat: Primary Malignant Tumors of the Lung, Their Incidence in the Philippines and Their Most Common Clinical Manifestation. *J. Philippine Med. Assn.*, 7:422–430, 1927.

Statistical Bulletin #31, #3, p. 8, March, 1950. Metropolitan Life Insurance Company.

Steiner, Paul E.: Incidence of Primary Carcinoma of the Lung with Special Reference to its Increase. *Arch. Path.*, 37:185–195, 1944.

Steiner, Paul E., E. M. Butt, and Hugh A. Edmondson: Pulmonary Carcinoma Revealed at Necropsy with Reference to Increasing Incidence in the Los Angeles County Hospital. *J. Natl. Cancer Inst.*, 11:497–510, 1950.

Weller, Carl V.: The Pathology of Primary Carcinoma of the Lung. *Arch. Path.*, 7:478–519, 1929.

Wells, H. Gideon: Cancer Statistics as They Appear to a Pathologist. *J.A.M.A.*, 88:399–403 and 476–482, 1927.

Winternitz, M. C., J. M. Mason, and F. P. McNamarra: The Pathology of Influenza. New Haven, Conn., Yale University Press, 1920.

Wolff: *Fortschritte der Medecine*, 1895, 13. (Reference from Sison and Monserrat.)

Wynder, E. L., and E. A. Graham: Tobacco Smoking as a Possible Etiologic Factor in Bronchiogenic Carcinoma. *J.A.M.A.*, 143:329–336, 1950.

A MODIFIED GREGG TYPE MICRORESPIROMETER*

GERALD R. SEAMAN

The need for a respirometer sensitive to the measurement of gas exchanges of the order of 0.05 mm^3 led to the use in this laboratory of a microrespirometer of the type described by Gregg (1947). Modifications of the originally described respirometer have been developed which have been found to increase the versatility and ease of use of the apparatus.

The essential features of the present form of the assembly† are diagrammed in Fig. 1. It consists essentially of a six arm capillary (I.D. approximately 0.5 mm) manifold. Each arm terminates at a standard taper 5/12 male joint (A) and is equipped with a stopcock (B) at its base. A fixed mark (C) is etched on the outside of each arm, midway along its length. Each arm also contains an index drop of Brodie's solution. Both ends of the manifold limb are equipped with 3-way stopcocks (D and E). The respiratory chamber proper (F) consists of the female portion of the standard taper 5/12 joint, flared to a bell, with a ground flange.

The manifold is connected by means of flexible plastic tubing to a fixed manometer, 45 cm. in height (I.D. 1.0 mm). The manometer is equipped with a pressure regulator (G) similar to that employed in the Cartesian diver apparatus (Holter, 1943). The entire manifold is fastened to a metal support (H) with the aid of metal fittings and thumbscrew (I). A rack and pinion assembly connects the metal support to the side of a constant temperature water bath, thus enabling the apparatus to be readily raised and lowered. A rectangular glass aquarium, 24″ x 10″, conveniently serves as the water bath.

The procedure for the filling of the respiratory chambers is as follows: A small, measured drop (in the region of 10 μl) of the respiring material, suspended in the desired incubation

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†The glass portion of the apparatus may be obtained from Scientific Glass Blowing Company, Houston, Texas.

mixture, is placed on a coverslip along with a bit of filter paper moistened with alkali. The coverslips are pre-treated with a coat of hydrophobic material, such as paraffin. Kirk type micropipettes (Sisco, *et al.*, 1941) are conveniently used for the delivery of the drops. The flared, ground, undersurface of the respiratory chamber is coated with stopcock grease (preferably a silicone preparation) and the chamber placed on the coverslip, enclosing the drop and filter paper. The ring of grease should be rather thick and must be discontinuous, in order to allow air to escape when the chamber is mounted. The capillary joint A is then lightly greased and the chamber with the attached coverslip is mounted onto the manifold. Care must be exercised during this operation not to jar the chamber, as this will result in the mixing of the respiring material with the alkali moistened filter paper. While the chambers are being attached to the manifold, stopcock D is closed and stopcock E is turned to connect the manifold to the manometer. The stopcock on the respective arm of the manifold is also open. During the attachment operation, positive pressure is applied with the regulator in order to maintain the index drop at a median position in the capillary arm.

After attaching all the respiratory chambers, the coverslips are carefully pushed flush with the flared surfaces of the chambers to assure a water tight connection, stopcocks D and E are opened to the atmosphere and the apparatus is submerged in the constant temperature and allowed to equilibrate for 5 minutes. Line J in Fig. 1 indicates the depth of submersion. After the equilibration period, stopcock D is closed and stopcock E is turned to connect the manifold to the manometer; B stopcocks are also closed. To take readings, the desired respiratory chamber is connected to the monometer by opening stopcock B on the appropriate arm and pressure (either positive or negative, as needed) is applied with the regulator (G) until the meniscus of the index drop in the capillary arm rests exactly on the etched mark (C). The drop is observed with the aid of a moveable, low power (10X) microscope mounted on the front of the water bath. After recording this pressure, stopcock B is closed and the next chamber is connected with the manometer.

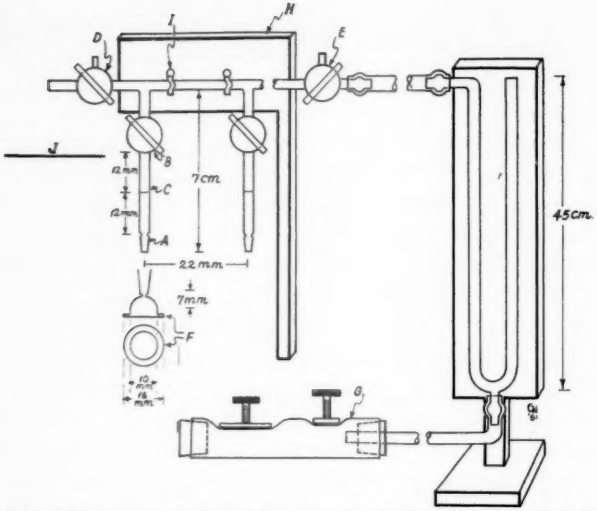


FIG. 1. Diagram of microrespirometer. Explanation in text. Not drawn to scale.

The rate of change of the pressure required to bring the index drop to the mark over a given period of time is the measure of the rate of gas utilization. The calculations for converting pressure readings into volumes of gas are identical to those commonly used with the Warburg manometer (Umbreit, *et al.*, 1949). The respiratory chambers may be readily calibrated by the method described by Scholander, *et al.* (1950). Variations caused by temperature and barometric pressure changes are corrected for by means of a blank respirometer which is used as a thermobarometer.

The use of ground joints to connect the respiratory chambers to the manifold allows for the interchangeability of chambers of various sizes and shapes. The sentitivity of the instrument may be readily changed by varying the dimensions of the respiratory chamber. Sensitivity of the apparatus may be further increased by constructing the manifold from capillary tubing of 0.1 mm I.D. By placing the reactants on a coverslip which is then attached to the respiratory chamber, loading of the chamber is made more convenient than was possible with the originally described apparatus.

The apparatus in its present form has been successfully employed for such manometric procedures as the determination of oxygen utilization, the production of carbon dioxide, the determination of succinic acid (Krebs, 1937) and of glutamic acid (Cammarata and Cohen, 1950) and many other methods which had been previously carried out in Warburg respirometers.

REFERENCES

Cammarata, P. S., and P. P. Cohen: *J. Biol. Chem.*, 187, 439, 1950.
Gregg, J. R.: *Rev. Sci. Inst.*, 18, 514, 1947.
Holter, H.: *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 24, 399, 1943.
Krebs, H. A.: *Biochem. J.*, 31, 2095, 1937.
Scholander, P. F., H. Niemeyer, and C. L. Claff: *Science*, 112, 437, 1950.
Sisco, R. C., B. Cunningham, and P. L. Kirk: *J. Biol. Chem.*, 139, 1, 1941.
Umbreit, W. W., R. H. Burris, and J. F. Stauffer: "Manometric Techniques and Tissue Metabolism", Burgess, Minneapolis, 1949.

THE SEARCH FOR NEW DRUGS AGAINST EPILEPSY*†

J. E. P. Toman, G. M. Everett and R. K. Richards,

Department of Pharmacology, Abbott Laboratories, North Chicago, Ill.

Introduction

This brief account is intended primarily to summarize the methodology and experiences of a pharmaceutical laboratory group engaged in the screening, assay, and evaluation of anticonvulsant drugs, together with some discussion of mechanisms of anticonvulsant action. For more systematic coverage of the field, several recent reviews are available (3, 13, 28, 31).

Approximately a million patients in the United States are afflicted with epilepsy, which is a generic term covering a variety of convulsive disorders usually characterized by intermittent attacks of impaired consciousness, by disturbances of motor, sensory, autonomic, or psychic functions, and by electrical signs of excessive and abnormal activity of the brain.

There are many possible classifications of the epilepsies (12, 22), but three distinct types of seizures are so frequently seen as to merit separate categories:

Grand mal, or major seizures—complete tonic-clonic convulsions accompanied by fast spike activity in the EEG (12).

Psychomotor, "epileptic equivalents," or psychical seizures—episodes of bizarre behavior or motor automatisms, psychical or complex sensory seizures, associated with slowly repeated EEG sharp waves, often with a temporal lobe focus (10, 22).

Petit mal—absences or "blackouts," myoclonic jerks, or akinetic falling seizures, associated with symmetrical 3 per second spike and wave discharges in the EEG (18).

Therapeutically, these seizure types usually respond differently to medication, even when different types occur in the same patient. For example, Dilantin [5,5-diphenylhydantoin] (20) and phenobarbital [5,5-phenylethylbarbituric acid] (14)

*A part of this material was presented in an exhibit at the 1950 meeting of the Federation of American Societies for Experimental Biology.

†A French translation of this article will appear in the "Revue due Centre Neuro-Psychiatrique".

protect primarily against grand mal seizures. Mesantoin [3-methyl-5,5-phenylhydantoin] (17) and Phenurone [phenylacetylurea] (11) have more general usefulness, but Phenurone in particular is active in suppressing psychomotor seizures. Tridione [3,5,5-trimethyloxazolidine-2,4-dione] (18, 25) and Paradione [3,5-dimethyl-5-ethyl-oxazolidine-2,4-dione] (5), find their greatest usefulness in the treatment of petit mal.

Although seizures can be reduced or abolished in a majority of patients by medication already available, new antiepileptic drugs are urgently needed because of the limitations of drugs now in use. Some of these limitations are:

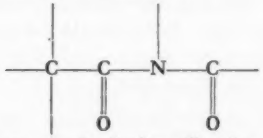
(1) Narrow range of usefulness (necessitating drug combinations)

(2) Low potency (necessitating large doses)

(3) Unpleasant side-effects (drowsiness, ataxia, excitement, gastro-intestinal upset, swelling of the gums, etc.)

(4) Occasional serious toxic effects (jaundice, blood dyscrasias, exfoliative dermatitis) (1).

The features of chemical structures which are responsible for these side-effects are relatively unknown. However, the structural requirements for antiepileptic action are better understood (4, 13, 15, 21, 24, 26, 31). For example, with the exception of bromide, most of the clinically effective antiepileptics have a common chemical root structure (24):



and those which are particularly effective against grand mal have a phenyl group on the first carbon.

In the search for new antiepileptics having higher potency, broader action, longer duration, and lower toxicity, approximately a thousand compounds, a large number of them having the above root structure, have been examined in our laboratory by methods now to be described.

Laboratory Screening Methods for New Anticonvulsants

Because of the variety of clinical types of epilepsy and their differences in response to drugs, it was thought important to

develop a battery of tests covering various aspects of anticonvulsant action, so that useful drugs would not be missed. Although there are already available a fair number of laboratory procedures for this purpose (3, 13, 15, 21, 27, 31), it is necessary in a screening program to sacrifice some thoroughness for speed and convenience. Therefore, during five years of experience the following three methods have been evolved for preliminary screening. They have the advantage that a large number of new compounds can be scanned in a short time. Four to six drugs can be handled by a single technician in the course of a morning or afternoon.

White mice, 20 to 25 gm., are used routinely because of their convenience in handling, although fatalities after convulsions are high in comparison with larger species. No animal is used again for a week after a previous test. Drugs are given orally, since anticonvulsants are ultimately intended for chronic oral use in patients. The drugs are made up as 2 per cent solutions or homogenized suspensions in tragacanth. For most drugs twelve mice are tube-fed doses of 200, 400, and 800 mg./kg., with four mice at each dose level. For the next hour the mice are examined for changes in behavior, such as ataxia, sedation, excitement, etc. They are then subjected to the following tests:

Metrazol test (4, 6, 8, 15, 27): Two mice on each dose level are given Metrazol 100 mg./kg. subcutaneously as a 1 per cent aqueous solution, and are observed closely over a thirty minute period. All control mice show a sequence consisting of excitement, isolated myoclonic jerks, clonic seizures, and finally severe tonic convulsions ending fatally. Increasing doses of effective drugs abolish these signs in the reverse of the order given. For convenience, the abolition of both tonic and clonic seizures is taken as the endpoint for determining the effective dose.

(In principle, this test is basically a measurement of threshold for drug-induced seizures.)

"Psychomotor" test (9, 29, 30): The other two mice on each dose level are subjected to low frequency stimulation producing seizures resembling psychomotor attacks in man. Rectangular pulses of 6 per second frequency, 1 msec. pulse width, and a

current of 50 mA (approximately 4 times threshold) are delivered for 3 seconds through a rigidly mounted pair of cup electrodes filled with salt paste, against which the eyes are gently pressed. The ensuing seizures are subtle in character and require close observation. Following the shock, normal animals show at least 10 and on the average 25 seconds of abnormal behavior, most simply described as "stunning." They preserve an awkward stance and do not respond normally to painful or other stimulation by walking or running. The face and forelimbs are frequently involved in rhythmic automatisms. Righting reactions are preserved. Abruptly at the end of the seizures the animal resumes normal locomotion and exploratory behavior. The most effective drugs permit the animal to walk away promptly after the shock. For a convenient end-point, the animal is considered protected if he walks normally within 7 seconds after the end of the shock.

(In principle, this test is one of *electrical threshold*, and is related to previously described threshold tests (13, 27, 31).

Supramaximal electroshock test (31, 32): After at least a fifteen minute interval, the same animals previously used for the psychomotor test are given a supramaximal shock, using the same technique but with a pulse frequency of 100 per second, pulse width 1 msec., current 100 mA (approximately 5 times threshold) and duration 0.33 seconds. The normal seizure pattern is characterized by a severe tonic phase of 14 seconds average duration, with an initial brief flexion of the limbs for 2 seconds followed by a longer period of complete extension, and terminating in a few clonic jerks. Unlike larger species, mice frequently die of anoxia at the end of the tonic phase unless given artificial respiration. Surviving animals show a typical severe postseizure depression. Effective drugs in increasing doses first abolish the tonic extensor phase, then the entire tonic phase, leaving a clonic or running type of seizure, or a "stunning" or psychomotor type of attack. As a practical end-point of protective action, abolition of the entire tonic phase is noted.

(In principle, this test is a measure of *qualitative responsiveness*, rather than of quantitative change in threshold, to electrical stimulation.)

If necessary to complete the preliminary screening, lower doses are tested, each 50 per cent of the previous dose, until an ineffective level is found. Doses higher than 800 mg./kg. are usually not tried, since drugs of such low potency are probably not clinically practicable.

Interpretation of Results

To determine a *protective index*, the average dose required to produce minimum neurological disability can be divided by the average effective doses in each of the three tests. If the protective index is much greater than 1.0 by one or preferably more of these tests, further determinations of the effective and toxic doses are made in mice and other species. Duration of action, chronic toxicity and other properties are studied before considering clinical trial.

In Table I are plotted some results obtained by this battery of preliminary screening tests in a series of drugs of known clinical value. A study of the table shows that with any one test alone valuable drugs might have been missed, while in all instances the useful drugs showed anticonvulsant action at non-toxic dosages by one or more tests. Although not shown in the table, experimental drugs have been found which were active by only one of each of the three tests, showing that they give relatively independent information. These results confirm the importance of using a battery of tests.

Correlation of Clinical and Experimental Findings

Table I shows that no single test can be relied upon to predict specificity of drug action, and also that quantitative comparisons of protective index may have little value, especially when those indices are less than 1.0. Originally these tests were developed in the expectation that their results might correspond to therapeutic results in the three categories of clinical seizures. However, this has not proved to be the case. For example, although most drugs clinically effective against grand mal are active by the maximal electroshock test, there are important exceptions. Figure I is an attempt to show in a diagrammatic way the qualitative relationship between the three laboratory

TABLE I

Comparison of Some Clinically Useful Antiepileptics By Three Screening Tests

$$\text{Protective Index} = \left(\frac{\text{Mean minimal toxic doses}}{\text{Mean protective dose}}\right)$$

Drug	Supramaximal Electroshock	Metrazol	Psychomotor Electroshock	Chief Clinical Use
Phenobarbital (5,5-phenyl ethyl barbituric acid)	0.7	1.7	<0.25	GM
Dilantin (5,5-diphenyl hydantoin)	3.0	0	0	GM
Mesantoin (3-methyl-5,5-phenyl ethyl hydantoin)	2.0	1.5	0.71	GM, PS
Tridione (3,5,5-trimethyl oxazolidine-2,4-dione)	0.2	1.7	0.47	PM
Phenurone (phenylacetylurea)	2.3	3.5	1.3	PS, GM, PM

tests and clinical convulsive disorders. It can be seen from results with these better known drugs that the tests have a potential predictive value concerning clinical usefulness and specificity, particularly if the drugs are active by more than one laboratory test.

MECHANISM OF SEIZURES AND OF DRUG PROTECTION

In addition to the practical screening of new drugs, considerable attention has been given to theoretical aspects of seizures and of drug protection against them, utilizing electrical stimulation of brain and isolated nerve preparations. From these studies and clinical evidence the following tentative conclusions have been drawn:

A. In individual neurones: Bursts of rapid repetitive excessive electrical stimulation lower the threshold and elicit high frequency discharges (up to 1000 per sec.): This activity in some cells can "detonate" others to which they project, leading to propagation of convulsive activity (2, 28).

B. In circumscribed cortical areas: Excessive single shocks can produce simultaneous discharge of elements which normally respond in a timed synaptic sequence, increasing the effective output of the system, which in turn can hypersynchronize other areas (29, 30).

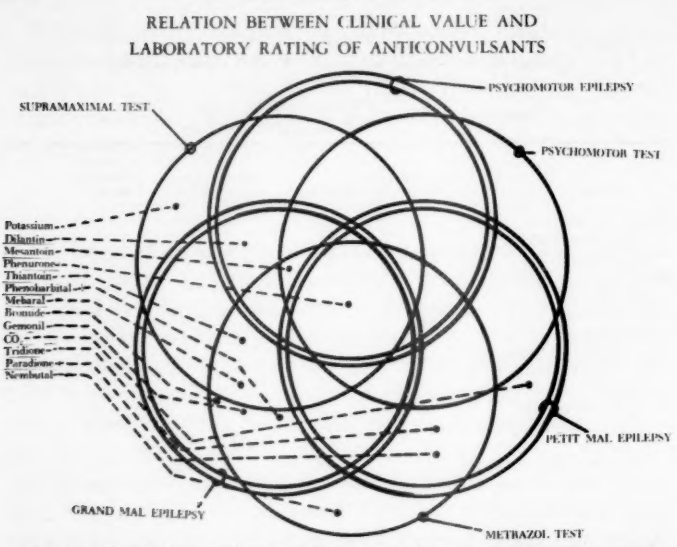


FIG. I. Correlation between clinical usefulness and laboratory results with common antiepileptics. The double circles enclose drugs effective in one or more of the three clinical categories. The single circles enclose drugs effective in one or more of the three laboratory tests. The diagram shows that the classifications of drugs by clinical usefulness and by laboratory tests are not congruent, but are overlapping and related.

Through mechanism A the entire brain can be successively detonated from various initial foci to produce a major convulsion; clinical grand mal seizures are probably the end result of this process (2, 31).

Through mechanism B a focus of convulsive activity can drive projection areas without detonating them, resulting in a disruption of their activity. This mechanism probably operates in many clinical psychomotor seizures from a temporal lobe focus (10), in petit mal from a subcortical medial focus (16), and in various types of aura and prodromata from other foci (22).

Two distinct mechanisms of action of anticonvulsant drugs have been determined experimentally (9, 28, 29, 30, 31). The first, typified by Dilantin, protects neurones against the development of hyperexcitability and abnormal discharge produced by excessive stimulation. The second, illustrated by Tridione, involves an increase in threshold and associated changes which

prevent hypersynchronization. Both mechanisms are shown by Phenurone and to a lesser extent by phenobarbital.

Discussion and Conclusion

In the course of this combined program of practical and theoretical research in anticonvulsant drug action, over a thousand drugs have been screened, of which five have already proved of value in clinical epilepsy:

Tridione—petit mal (8, 18, 23, 24, 25)

Paradione—petit mal (5, 8a)

Gemonil—grand mal with demonstrable cerebral lesions (6, 22a)

Phenurone—chiefly psychomotor, but also grand mal and petit mal (7, 11)

Nuvarone—grand mal (8a, 19)

Several more drugs are in the process of clinical evaluation, and others are under consideration for eventual trial. The remainder have given valuable leads concerning structure-activity relationships and mechanisms of action, providing a basis for the synthesis of new compounds.

It is conceivable that the time may come when surgical and pharmacotherapeutic methods will have advanced to a point where practically complete control of epileptic seizures can be achieved. Clinical experience with the best available therapy shows that freedom from attacks can often be attained without unwanted side-reactions. But our present armamentarium of drugs and surgical procedures is not yet large enough. Progress will surely be more rapid when our laboratory methods, still largely empirical, are either reinforced or replaced through a deeper theoretical understanding of the mechanisms of seizures and of their prevention.

REFERENCES

1. Abbott, J. A., and R. S. Schwab: *New England J. Med.*, 242:943, 1950.
2. Adrian, E. D., and G. Moruzzi: *J. Physiol.*, 97:153, 1939.
3. Bertrand, I., Th. Gayet-Hallion, and D. Quivy: *J. Physiologie* (Fr.), 41:499, 1949.
4. Cheymol, I., in "Actualités Pharmacologiques" by R. Hazard: Masson. Paris, 1950.
5. Davis, J. P., and W. G. Lennox: *Res. Publ. Assoc. Nerv. Ment. Dis.*, 26:423, 1947.

6. Everett, G. M.: *Proc. Fed. Am. Soc. Exp. Biol.*, 5:26, 1946.
7. Everett, G. M.: (Presented before Am. Br. Internat. League Against Epilepsy, June 13–14, 1948).
8. Everett, G. M., and R. K. Richards: *J. Pharmacol. and Exper. Therap.*, 81:402, 1944.
8a. Everett, G. M., and R. K. Richards: *Proc. Fed. Am. Soc. Exp. Biol.*, 9:270, 1950.
9. Everett, G. M., and J. E. P. Toman: *Proc. Fed. Am. Soc. Exp. Biol.*, 10:293, 1951.
10. Gibbs, E. L., B. Fuster, and F. Gibbs: *Arch. Neurol. Psychiat.*, 60:331, 1948.
11. Gibbs, F. A., G. M. Everett, and R. K. Richards: *Dis. Nerv. System*, 10:47, 1949.
12. Gibbs, F. A., and E. L. Gibbs: *Atlas of Electroencephalography*, Lew A. Cummings Co., Cambridge, Mass., pp. 221, 1941.
13. Goodman, L. S., J. E. P. Toman, and E. A. Swinyard: *Arch. Int. Pharmacodyn.*, 78:144, 1949.
14. Hauptmann, A.: *München Med. Wochnschr.*, 59:1907, 1912.
15. Hazard, R., and J. Cheymol: *J. Pharmacy and Pharmacol.*, 2:1, 1950.
16. Jasper, H. H., and J. Droogleever-Fortuyn: *Res. Publ. Assoc. Nerv. Ment. Dis.*, 26:272, 1947.
17. Kozol, H. L.: *Am. J. Psychiat.*, 103:154, 1946.
18. Lennox, W. G.: *J.A.M.A.*, 129:1069, 1945.
19. Livingston, S., and A. Y. Sweet: *Bull. Johns Hopkins Hosp.*, 86:359, 1951.
20. Merritt, H. H., and T. J. Putnam: *Arch. Neurol. Psychiat.*, 42:1053, 1939.
21. Merritt, H. H., and T. J. Putnam: *Epilepsia*, 3:51, 1945.
22. Penfield, W., and T. C. Erickson: *Epilepsy and Cerebral Localization*, Springfield, Ill., Charles C. Thomas, 1941.
22a. Perlstein, M. A.: *Pediatrics*, 5:448, 1950.
23. Richards, R. K., G. M. Everett, and K. L. Pickrell: *Anesth. and Analg.*, 25:147, 1946.
24. Richards, R. K., and G. M. Everett: *J. Lab. and Clinic Med.*, 31:1330, 1946.
25. Richards, R. K., and M. A. Perlstein: *Arch. Neurol. Psychiat.*, 55:164, 1946.
26. Spielman, M. A.: *Proc. First Nat. Medicinal Chem. Symp. of the Am. Chem. Soc.*, 119, June 17–19, 1948.
27. Swinyard, E. A.: *J. Amer. Pharmaceut. Assoc.* (Sci. Ed.), 38:201.
28. Toman, J. E. P.: *J.E.E.G. Clin. Neurophysiol.*, 1:33, 1949.
29. Toman, J. E. P.: *Neurology*, 1:444, 1951.
30. Toman, J. E. P.: *Proc. Fed. Am. Soc. Exp. Biol.*, 10:137, 1951.
31. Toman, J. E. P., and L. S. Goodman: *Physiol. Rev.*, 28:409, 1948.
32. Toman, J. E. P., E. A. Swinyard, and L. S. Goodman: *J. Neurophysiol.*, 9:231, 1946.

THE SURGICAL TREATMENT OF INVOLUNTARY MOVEMENTS*

A. Earl Walker

It is a distinct privilege and honor to present the James Greenwood lecture in Neurosurgery. The man for whom this lectureship was named, delved into many aspects of nervous and mental disease, but he apparently had a particular interest in the physiology of movement. For in his select bibliography is a paper (Greenwood, 1935) read before the section on neurology and psychiatry of the Southern Medical Association in 1934 on "The mechanisms of the corpus striatum and the cerebellum in muscular action." In this paper Dr. Greenwood discussed the influence of what we now know as the extrapyramidal system upon the corticospinal tract which mediates volitional movement. I feel sure that he would be fascinated by the changing concepts which have developed in the past two decades, and that he would attend with interest the story of our surgical attempts to solve the riddle of abnormal movements.

The grotesque appearance of a person exhibiting involuntary uncontrollable movements is a spectacle to excite both sympathy and pity. But through the ages, the physician, barber surgeon or medicine man was unable to offer much relief to the abject sufferer by herbs, "mummy," blood letting or incantation. To these forlorn creatures, Victor Horsley, rugged pioneer surgeon of the nervous system, offered the first ray of hope. He had explored with the electric current, the brain of every animal he could lay his hands on by hook or crook.[1] Upon this extensive experimental background Victor Horsley based his clinical researches on the surgical relief of involuntary movements. It is

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*The James Greenwood Lecture, The University of Texas Medical Branch, Galveston, October 3, 1951. From the Division of Neurological Surgery, The Johns Hopkins University Medical School, Baltimore, Maryland.

[1]It is related that Horsley was so intent upon extending his experiments on cortical stimulation to every known animal, that he occasionally "borrowed" from his friends pets on which he would operate, stimulate the cortex, close the wound and return the animal to its owner. This habit was not conducive to improving rapport, and occasionally, if the animal did not survive the experience, Horsley lost a friend.

in accord with the character of this great man that the initial report of cortical resection for involuntary movements was given, not as a spectacular surgical triumph, but in a paper on the functions of the motor cortex.

In the Linacre lectures of 1909, Horsley (1909) modestly described how he operated upon a boy who suffered from athetosis of the left arm, how he located the area of the cerebral cortex which, on electrical stimulation, gave rise to arm movements and how he excised it by subpial resection (Fig. 1). Thirteen

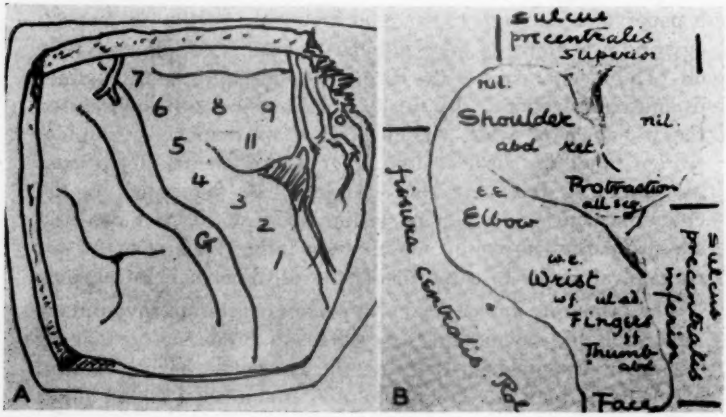


FIG. 1. Horsley's sketch of the operative field (A) and cortex removed (B) of his celebrated case of athetosis. The results of stimulation at the points indicated (A) follows:—

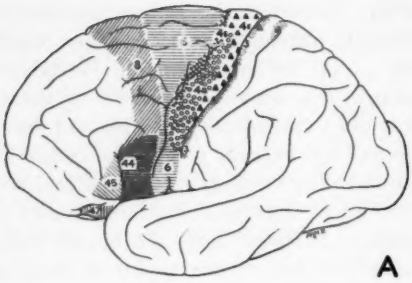
1. Movement of the left side of the face.
2. Abduction of the left thumb, and to this on repetition of the excitation was added flexion of all the fingers.
3. Flexion of the fingers, flexion of the wrist with ulnar adduction and late flexion of the elbow.
4. Extension of the fingers ulnar abduction of the wrist, questionable flexion of the elbow.
5. Extension of the wrist elbow held at a right angle in "confusion".
6. The same movement as 5, and in addition abduction of the shoulder.
7. No movement of the upper limb.
8. Elbow held at a right angle, powerful retraction of the shoulder.
9. Elbow at a right angle protraction of the arm, and extension of the wrist.
10. Nil.
11. Protraction of the arm, elbow at an obtuse angle, extension of the writ, and questionable extension of the fingers.

The abbreviations used on the specimen (B) are as follows:—Abd., abduction; ret., retraction; e.e., elbow extend; w.e., wrist extend; w.f., wrist flexed; ul. ad., ulnar adduction; f.f., fingers flex.

months after this operation, the patient was still free of involuntary movements although, as Horsley emphasized, he had regained some power in the left arm. Probably because the surgical procedure was not expounded for its therapeutic possibilities, it was only sporadically carried out in the next few years. Yet it was not entirely forgotten. A few decades later, when interest in the physiology of the cerebral cortex revived, largely due to the neurophysiological researches carried on in the laboratories of Professor John Fulton of Yale University, surgeons recalled Horsley's report and were stimulated to tackle again the problems of motor disturbances.

Since the pioneering investigations of the latter part of the nineteenth century, surgical and physiological techniques had been improved and important anatomical researches had been made on the cerebral cortex. At this point it may be well to digress and discuss the changing concepts of cerebral anatomy and physiology. It was apparent to the early anatomists that the cerebral cortex was not a uniform mantle but that its thickness and composition varied. In 1905 Alfred Campbell gave a detailed description of the cytoarchitectural areas of the cerebral cortex. His map was the stimulus for an unprecedented study of cerebral architectonics. The investigations of Brodmann (1909), Economo and Koskinas (1925), the Vogts and their pupils (1919) were so detailed and diverse that the borders of cytoarchitectural areas changed time after time. With each new investigator subdividing the original divisions, it seemed that only the number of cortical neurones would limit the minute areas. It was natural that there should be a reaction to this microscopic parcellation. Lashley and Clark (1946), in reviewing the cortex of the monkey, found that only a relatively few types of cortex could be consistently recognized and few boundaries could be defined with any degree of accuracy. von Bonin and Bailey (1947) have further emphasized these difficulties in the older maps. On the most recent map of the human cortex von Bonin (1949) indicates the following areas in the precentral region (Fig. 2).

The concepts of the physiology of the motor cortex were also undergoing changes. The general schema of the early investigators (Ferrier, 1876) was confirmed but certain elaborations



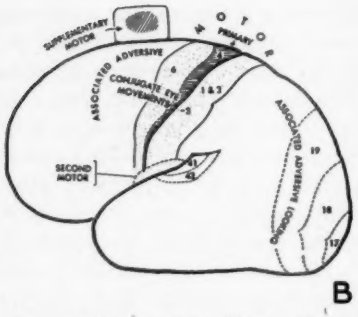


FIG. 2. von Bonin's cytoarchitectural map of the precentral region (A) and Penfield and Rasmussen's motor map of the human cerebral cortex (B).

Area 4 γ, the gigantopyramidal cell area, or area 4 of lower animals, gives rise to the large fibers which comprise about 10% of the corticospinal tract.

Area 4a, is similar to area 4 but has no cells of Betz.

Area 4 s, is characterized by the presence of large pyramidal cells in the fourth layer.

Area 6, is an agranular area having a columnar pattern.

were added. Marion Hines (1937) found that stimulation of a strip of cortex in the anterior part of the excitable motor cortex produced an inhibition of contracted skeletal muscle on the opposite side and extirpation of that band, spasticity in those muscles. The concept derived from these data was further elaborated by Dusser de Barenne, *et al.* (1941) on the

basis of strychnine neuronography and incorporated in a hypothetical suppressor system which included other suppressor bands in the frontal and parietal lobes. Based on this theory, rather elaborate schemata of cortical and subcortical organization were proposed. But doubt has been cast on the existence in the the monkey of these bands in the form originally postulated. In man there is no evidence of such a suppressor system although undoubtedly inhibition may be obtained by stimulation of certain cortical areas. A more important development was the elucidation of second motor and sensory areas in the cerebral cortex by Adrian (1940). These findings have been elaborated in animals by Woolsey (1947) and in man by Penfield, *et al.* (1950). The recent map by Penfield and Rasmussen indicates the present concept of the organization of the motor cortex (Fig. 2). It perhaps should be emphasized that the pre- and post-central convolutions are equipotential motor areas and give rise to similar responses on electrical stimulation. The anterior lip of the central sulcus is differentiated from the general area by its lower threshold of response.

Researches upon the basal ganglia were demonstrating that they acted upon a dynamic or kinetic motor pattern. In the absence of a posture or movement, stimulation of the striatum caused no discernible response, but Tower (1936) found that if the musculature was actively innervated, excitation of the striopallidum produced vigorous inhibition. Mettler, *et al.* (1939), confirmed this effect on movements electrically induced from the cerebral cortex. The strychnine neuronography experiments of Dusser de Barenne, *et al.* (1941), suggested that certain areas of the cortex (their "suppressor bands") had an influence on the striatum which was interpreted as playing a role in the inhibition. Since the connections of the striatum to the thalamus by way of the ansa lenticularis had been long established, it was logical to postulate a cortico-striothalamocortical circuit ending in cortical areas 4 and 6 which might modify motor performance.

It was natural as some insight was obtained into the functioning of the cerebral cortex and basal ganglia, that attempts should be made to schematize the mechanisms involved in the different involuntary movements. It is probable that these

schemata are based upon inadequate and unproved data and tend to over-simplify the physiological phenomenon. The evidence derived from the clinical experiments does confirm one generalization, early suggested by Kinnear Wilson, namely that tremor is dependent largely on the integrity of the corticospinal or pyramidal tracts; whereas choreoathetosis is related in great degree to the activity of extrapyramidal systems.

Surgical Techniques

The development of the surgical procedures for the relief of involuntary movements has proceeded pari passu with the evolution of the knowledge of cerebral functions.

Cortical Operations

The idea that removal of the cortical motor area might eliminate involuntary movements apparently was prevalent about the turn of the twentieth century for Frazier (1906) relates in a paper published in 1906 that a boy suffering from athetosis had had a craniotomy performed and the cortical center of his left arm excised. The operation apparently did not alleviate the condition. Unfortunately, no details of the technique used are given.

But it was Horsley's demonstration that resection of the motor cortex could abolish athetosis, that stimulated interest in the subject. Anschütz (1910), one year after Horsley's paper appeared, reported a case of athetosis in which he removed the cortical "arm center" with abolition of the movements. About a decade later Payr (1921) demonstrated to the Leipzig Medical Society a patient whose athetosis had been relieved by undercutting the corresponding cortical field. In 1927 Nasaroff mentioned several cases treated by Russian surgeons according to Horsley's technique, and he reported two cases in which alcohol had been injected in the motor arm area for athetosis with relief. But neither case had been followed more than a few months. Naffziger (1937) about the same time tried intracortical injection of alcohol in four cases with improvement only in one case. Polenow (1929) also relieved abnormal movements by a cortical operation.

But the surgical treatment of motor disturbances received little attention until the physiology of the cerebral cortex was re-investigated by Fulton and his coworkers. Based upon the new concepts of cerebral physiology, Paul C. Bucy made cortical resections for the relief of involuntary movements. In his early cases Bucy (1932, 1937) removed the excitable cortex corresponding to the involved member to the lateral ventricle. His later extirpations (1942) were planned to include only certain cytoarchitectural areas in front of the central sulcus. As it became apparent that tremor was related to the functioning of the corticospinal system, Bucy (1947) suggested that an extirpation limited to the posterior half of the pre-central gyrus (area 4) might abolish it. However, experience proved that this limited ablation only depressed the tremor. In a recent communication Bucy (1947) expressed the view that "extirpation limited to the pre-central gyrus (area 4) suffices to abolish tremor" but that "it requires a much more extensive extirpation of the precentral motor cortex to abolish the movements of choreoathetosis." To relieve the latter he removed the pre-central gyrus including the anterior wall of the Rolandic fissure and a portion of the frontal gyrus lying anterior to it (Fig. 3).

In 1937 Klemme began a series of "premotor cortical" resections for involuntary movements, but he has never described his operative technique in precise terms. Accordingly the site and extent of his ablations are unknown, except that they were said to be anterior to the motor (area 4) cortex.

As the concept of the suppressor system gained recognition, surgeons were stimulated to relate their resections to the suppressor area. Reid (1948) argued that the suppressor strip was under the influence of area 6, which, if removed, should allow greater inhibition of subcortical structures, and hence relief of the involuntary movements. Accordingly, he endeavored to map out the motor area and area 4 s by the effect of electrical stimulation of the motor cortex on electrographic tracings and the tremor. He stated, "If stimulation to a particular point abolishes the tremor and the spontaneous electrical activity in the cortex for about ninety seconds, the point is regarded as corresponding to a point in the suppressor strip, area 4 s . . . .

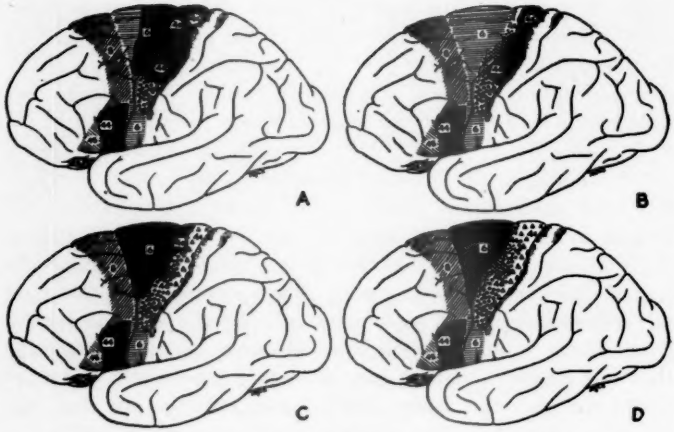


FIG. 3. Diagrams to illustrate types of cortical resection used by

A. Bucy for choreoathetosis.
B. Bucy for tremor.
C. Klemme for dystonia.
D. Reid for tremor.

This region is regarded as being the posterior limit of the area to be removed . . . The remaining three borders of the area are marked out by anatomical inspection. The lower limit of removal corresponds to the level of the face area in area 4 except on the side of the motor speech area in which the resection is not extended below the level of the arm area. The medial border of resection is the midline, and the anterior border is gauged on morphological ground being, as far as one can judge, the junction between areas 6 and 8." This procedure did not give consistently good results. Barry Wyke (1950) who assisted Reid assumed that the interruption of fibers between areas 4 s and 6 was the important factor in the relief of the tremor and rigidity. Upon the basis of his verbal report, before the published paper of Reid was available, a number of American neurological surgeons made a deep cut presumably between these areas in some nine cases of Parkinsonism without appreciably altered the tremor or rigidity in more than one case. Moreover, in only one of these instances was it possible for the

operator to determine a suppressor point by the effect of electrical stimulation on the electrocorticogram or the tremor. It was true that the tremor could be abolished by electrical stimulation of the motor cortex (area 4) but it was not possible to define a strip anterior to it, stimulation of which abolished the movements. At about the same time Meyers *et al.* theorized that a cut between area 4 and 4 s might be effective in relieving the involuntary movements of hemiballismus (Fig. 4). He carried

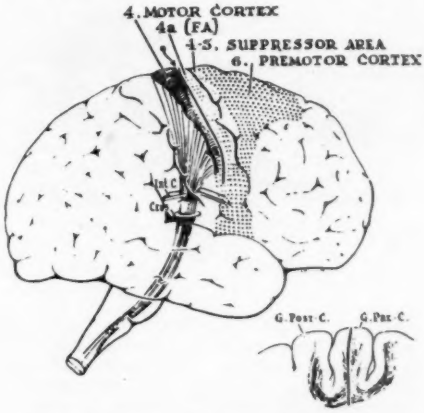


FIG. 4. Diagrammatic sketch to illustrate Meyers et al.'s incision between areas 4 and 4s for the relief of hemiballismus.

out this procedure in two cases, but in neither was the suppressor band clearly demonstrated. One patient, unfortunately, did not survive more than a month; the other made a complete recovery.

CAPSULAR OPERATIONS

Meyers (1942) made a direct attack upon the basal ganglia to relieve the tremor and other symptoms of paralysis agitans. In his first four cases the head of the caudate nucleous was extirpated and certain of the extrapyramidal and corticopetal fibers of the anterior limb of the internal capsule were interrupted. Although the tremor was decreased, the rigidity was less affected. In the next two cases the oral part of the putamen and

globus pallidus was removed in addition and the fibers in the oral ¾ of the anterior limb of the internal capsule were sectioned. Tremor was said to be abolished in these two cases and rigidity decreased. However, to preclude vascular damage to the posterior limb of the internal capsule, Meyers modified his procedure interrupting the pallidofugal fibers after removal of the head of the caudate nucleus (Fig. 5). To Jefferson Browder

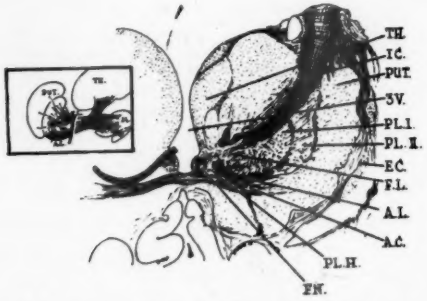


FIG. 5. To illustrate Meyers' pallidofugal operation. The general anatomical relations at the level of the anterior commissure are shown. The broad line in the larger figure indicates the first incision, and that in the insert the second. The abbreviations are as follows:—A.C. anterior commissure; A.L., ansa lenticularis; E.C., external capsule; F.L., fasciculus lenticularis; FN., fornix; I.C., internal capsule; M., midbrain; Pl. I., globus pallidus, crus I; Pl. II., globus pallidus crus II; Pl. H., pallido-hypothalamic fibers; Put., putamen; Th., thalamus; 3 V., third ventricle.

(1947) who had followed Meyers' earlier cases, the essential part of this technique was the interruption of the anterior limb of the internal capsule rather than the section of the pallidofugal fibers. Through an incision parallel to and 3 cm. from the falx just anterior to the premotor area Browder (1948) entered the ventricle and removed the head of the caudate nucleus to expose the inner fibers of the anterior limb of the internal capsule. With a blunt, right-angled hook the section was started at the rostral end of the capsule just short of the corona radiata. As the fibers were cut the surgeon noted the amplitude

of the tremor and grip of the contralateral side. At approximately the knee of the capsule the tremor became abolished but Browder found that to obtain lasting relief the procedure had to be continued until there was such a marked paresis of the hand that the patient was just capable of slight flexion of the fingers (Fig. 6).

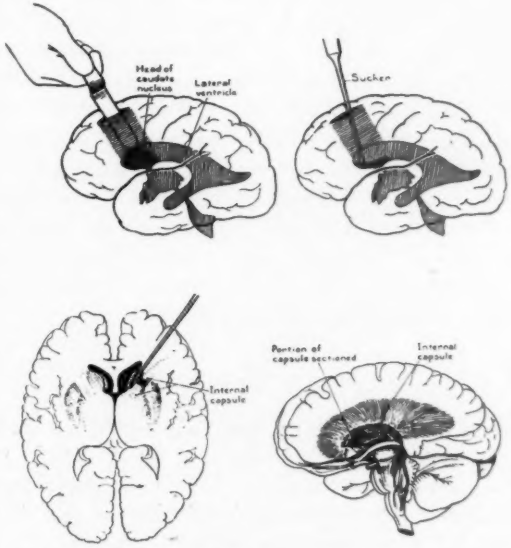


FIG. 6. Sketches to show Browder's technique of capsulotomy for the relief of tremor.

Another type of attack has been made upon the basal ganglia by Spiegel and Wycis (1950) using a stereoencephalotome. With this instrument electrolytic lesions have been placed in the globus pallidus and thalamus for the relief of involuntary movements.

CEREBRAL PEDUNCULOTOMY

From the physiological studies of tremor and the clinical results of surgical procedures for its alleviation, interruption of the corticospinal tract seemed essential for its abolition. But the

cortical, capsular and spinal operations all had certain disadvantages. As the result of a search for a level at which the corticospinal tracts are relatively isolated, superficial and accessible to surgical approach, the cerebral peduncle was selected by the author as a favorable operative site (Walker, 1949). At this point the corticospinal tract could be sectioned with little danger of injury to other structures which might complicate the clinical result. Moreover, since it seemed likely that many extrapyramidal motor pathways did not pass through the peduncle, there remained the possibility that some useful movement might be retained or recovered after the operation.

The operation (Walker, 1949) has been carried out through a subtemporal approach. The base of the brain is exposed and an incision 6–7 mm. in depth is made across the outer 2/3 to 3/4's of the cerebral peduncle (Fig. 7). If the position of the corticospinal tract is located by electrical stimulation, the cut is carried through the peduncle until just a feeble muscular response in the arm or face can be produced.

Spinal Operations

Section of the spinal roots was early advocated for the correction of spasticity but Winslow and Spear (1912) were the first to cut the posterior roots and relieve athetosis in one leg. One wonders if the movements in Winslow's and Spear's patient were not a manifestation solely of spasticity for neither Frazier (1918) nor Foerster (1921) were able to abolish choreoathetosis by posterior root section; nor did Puusepp (1930), Foerster and Gagel (1932), and Pollock and Davis (1930) influence the tremor of paralysis agitans by dorsal rhizotomy, although rigidity was diminished. Puusepp (1930) and Rizzatti and Moreno (1936) could not abolish the shaking palsy by section of the posterior columns of the spinal cord. Neither did Foerster, Putnam (1938) or Oldberg (1938) affect it by anterolateral cordotomy; yet Mashansky (1935) reported three "permanent" recoveries and five "fairly satisfactory" results in a series of 17 patients having alternating tremors and other abnormal movements! But this procedure—cutting the vestibulospinal, reticulo-spinal and tecto-spinal tracts at the level of the

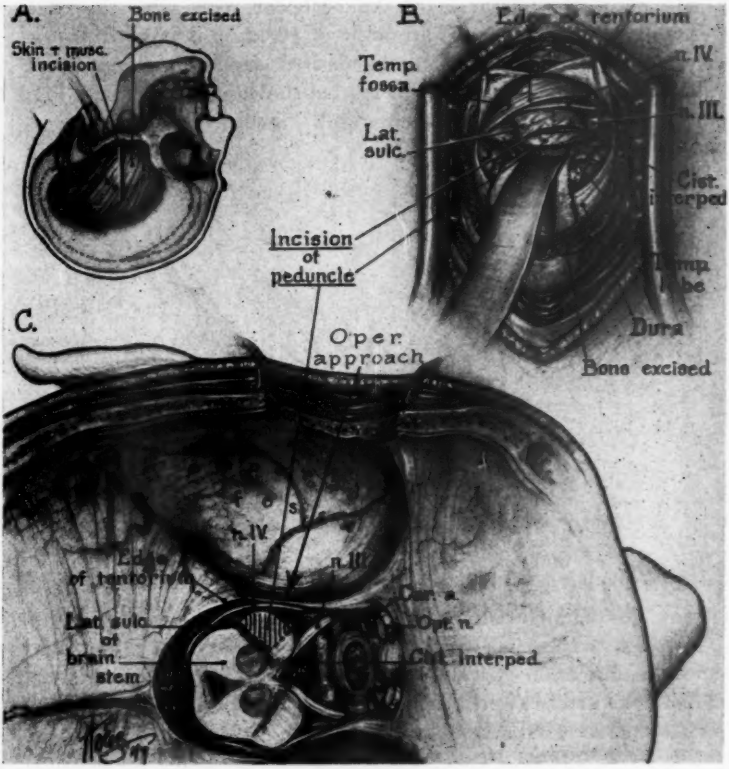


FIG. 7. The author's operative approach for section of the cerebral peduncle. A. The head hangs down to allow the temporal lobe to fall away from the floor of the middle fossa. B. By gentle retraction the incisura tentorium is exposed and the arachnoid incised over the brainstem. The incision 6 to 7 mm. in depth is made from the lateral sulcus medially. C. A sketch to show the relations in the operative field. The technique used by Guiot and Pecker is similar except that the incision is only 3 mm. in depth.

second cervical vertebra—as carried out by Putnam did alleviate the movements of choreoathetosis. To relieve tremor, Putnam devised a section of the lateral pyramidal tract in the cervical region. After marking a depth of 4 mm. on a sharp pointed blade, the tip of the knife was inserted just lateral to

the point of emergence of the posterior roots with the edge pointed downwards. At an angle of about 10 degrees from the vertical it was inserted to the desired depth and then swept laterally so that the point emerged at the dentate ligament (Fig. 8). This procedure has been slightly modified by other opera-

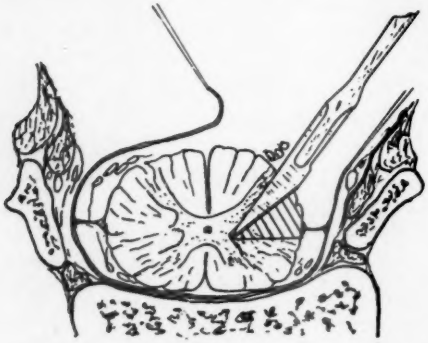


FIG. 8. Diagram showing Putnam's technique of pyramidotomy at the second cervical segment of the spinal cord.

tors, particularly Oliver (1949 and 1950) who has had a very extensive experience with spinal pyramidotomy. A rather radical modification was instituted by Ebin (1949) who sectioned both the crossed and uncrossed pyramidal fibers by continuing the cut across the midline to the anterior fasciculus (Fig. 9).

MISCELLANEOUS PROCEDURES

A number of other procedures have been tried at various times to relieve involuntary movement. Delmas-Marsalet and van Bogaert (1935) damaged the dentate nucleus of a patient suffering from a postencephalitic Parkinsonism. The rigidity was decreased but the tremor aggravated. Unfortunately, a thrombosis of the superior cerebellar artery complicated the clinical picture and the patient died ten days postoperatively.

On the basis of the effect of emotional disturbances on the tremor of Parkinsonism, prefrontal lobotomy has been performed without modifying the condition of the patient, except in

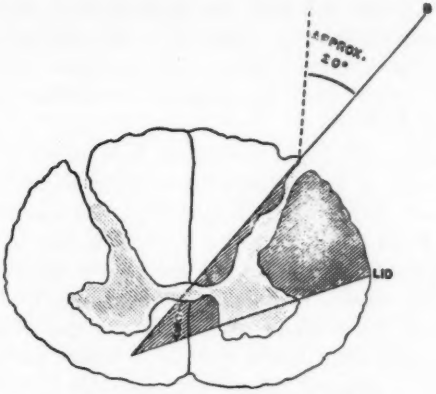


FIG. 9. Diagram to illustrate Ebin's technique of combined lateral and ventral pyramidotomy. The incision is 8 to 9 mm. in depth. The abbreviations are as follows:—TCSL, lateral corticospinal tract; TCSV, ventral corticospinal tract; LID, dentate ligament; B, line of incision, at angle of approximately 20 degrees with the vertical depth of incision, 8 to 9 mm.

one case reported by Rao and Morea (1949) in which there was immediate cessation of tremor and decrease in rigidity.

With a view of possibly improving the cerebral circulation, Gardner and Williams (1949) performed a variety of operations on the cervical sympathetic ganglia on both sides. Except for a slight euphoria of the patients, the different sympathectomies appeared to have no effect upon the manifestations of the disease.

RESULTS

The results of major neurosurgical procedures for the relief of involuntary movements can only be assessed several years after the operation. Too many factors tending to produce temporary improvement such as the psychological impact of a surgical procedure on a patient with a chronic disease, the diaschisis or neural shock of trauma to the central nervous system, and natural fluctuations in the disease, are operative in the immediate post-operative period to allow an early, true evaluation of surgical therapy. Deductions drawn from cases followed

from a few months to a year are likely to give rise to a spuriously enthusiastic opinion. Only by a detailed assessment of neurological, psychiatric, social and economic factors made at least two years after the surgical procedure can a valid conclusion be drawn.

This global evaluation of the patient is particularly important in the treatment of involuntary movements, which usually are but one phase of the patient's disability. Because of the other manifestations of the disease, it is rare to be able to restore the patient to a normal condition. In the case of the Parkinsonian syndrome, the rigidity and weakness may be as troublesome or more so than the tremor; in the case of the dystonias and athetoses, mental impairment may be so severe that even if the movements are stopped, the patient is unable to make a social adjustment. There is the added factor that many conditions characterized by tremor are progressive, so that new disorders appear as one is eliminated. For this reason the results of surgery for movements due to trauma or vascular lesions are better than those obtained when the motor disturbance is due to a chronic inflammatory or degenerative condition.

The surgery of the involuntary movements does not cure a disease; it merely arrests a symptom or symptoms of the illness. But even this is at a price, for all current operations for involuntary movements produce, at least temporarily, some degree of paresis. This may be so minimal that the patient hardly notices it or it may be so severe that the patient is seriously crippled. Perhaps the situation is not quite so desperate as Bucy states; "No permanent reduction in and certainly no permanent abolition of . . . tremor can be obtained without simultaneously producing a paralysis of the involved extremities of a variable and unpredictable severity." Although in occasional cases an ideal result—abolition of movement without discernible paralysis—may be obtained, it is certainly the exception rather than the rule. In younger individuals, the initial paralysis usually disappears to a considerable degree, at least sufficiently to allow ambulation, albeit with a limp, but in older people the hemiplegia may not regress.

From these comments it is obvious that the patients for operation must be rigidly selected. In the first place their move-

ments should be unilateral, for bilateral operations, even if practiced seriation, have been associated with a high mortality. In the second place, involuntary movement must be the most prominent disability for the other concomitants of Parkinsonism and cerebral injuries are little, if at all, benefited by operation. Finally the patients should be physiologically under the age of 50 years, for recuperation in older individuals is poor.

The mortality and morbidity has in general been relatively low. Klemme (1942) has reported a 17% mortality. Bucy (1950) has had no fatalities. From capsular operations six deaths occurred in a series of 25 cases (Browder, 1947). Hamby (1947) reported three fatalities following bilateral pallidofugal sections, three more following unilateral capsulotomy and one after a bilateral seriation section of the internal capsule in a total series of 17 cases. No deaths have been reported from cerebral pedunculotomy in the ten operated cases. Cordotomy seems to be well tolerated. Putnam (1938, 1940, 1942) has had no deaths in approximately 60 cases; Oliver (1950) five in 79 cases; Neimeyer (1945) one in nine cases.

I. *Choreoathetosis.* In this category all varieties of grotesque movement from severe dystonia musculorum deformans to mild post-hemiplegic athetosis are included. This type of movement is amenable in varying degree to the several types of operation. It was for this disorder that Horsley (1909) resected the excitable motor cortex. Experience has confirmed his report that, at least, the milder cases of choreoathetosis may be relieved by such ablations. The more severe cases may not be benefited by extensive destruction of the motor and premotor cortex. Bucy maintains that for the relief of choreoathetosis, the pre-central gyrus including the anterior wall of the Rolandic fissure and a portion of the frontal gyrus lying anterior to it must be removed. The efficiency of this procedure has been attested to by many surgeons (Alajouanine, *et al.* 1947, and Verbiest, 1949), but there are a number of cases in which the procedure has not proved effective. That some are due to imperfections in surgical technique as Bucy claims is probably correct.

Less extensive resections of the motor areas are said to relieve choreoathetosis. Klemme (1942), who does not separate

his cases of athetosis and tremor, maintains that his "premotor resection" will abolish the movements. However, since his results are presented in such vague terms, they cannot be critically evaluated. Other surgeons, stimulated by Klemme's enthusiastic reports, have not been able to obtain such good results with "premotor resection" except in isolated instances (Negrin, 1949, and Oldberg, 1938). The impression has been gained that perhaps a third of these patients were markedly improved with abolition of the tremor, but the majority were either not benefited or seriously crippled by the operation with or without modification of the tremor (Browder, 1947, and Money, 1948). Attempts to relieve choreoathetosis by lesions confined to the motor cortex have been said to reduce markedly the movement, but the follow-up has been very short. Hemiballismus, which probably should be considered a variety of choreoathetosis, was abolished by resection of the motor cortex in two cases reported by Alpers and Jaeger (1950) with a resulting hemiplegia. Meyers' *et al.* (1950) cases were relieved by an incision 2.5 cm. deep along the anterior margin of area 4.

Only one brief mention has been made of a pallidofugal operation for choreoathetosis. In the first few post-operative days, when hemiparesis was present, the movements were absent but as the strength of the limbs returned, the athetosis developed pari passu (Meyer, 1942). Cerebral pedunculotomy in two cases has not given satisfactory results. Hemiballismus, however, has been abolished with practically no paresis by pedunculotomy (Walker, 1949).

Putnam (1933 and 1938) has had the most extensive experience with cordotomies for choreoathetosis. His sections of the anterior columns in a series of more than 30 cases has given appreciable improvement in approximately two thirds of the cases. While no patient was relieved of the movements, they were sufficiently lessened that the patient was easier to handle. Oldberg (1938) reported ten cases on which he had performed anterior cordotomy with no deaths and only two failures, both in patients suffering from chronic encephalitis. However, the initial beneficial effects have been in most cases transitory and most surgeons have not been satisfied with the long term results.

II. *Tremor.* Tremor, most authors agree, may be eliminated by interruption of the corticospinal tract at any level. To effect a permanent relief by a cortical resection, Bucy states the precentral convolution must be removed. Neimeyer's (1945) observations on two cases would fall in line with that hypothesis. But Klemme (1942) maintains that premotor resection will abolish tremor. His report, previously discussed, merits no further comment. Reid's (1948) operative technique probably achieved much the same anatomical result as Klemme's, so that Reid's report is of particular interest. In none of his cases was the tremor completely abolished, although in a few instances it occurred only in times of excitement. Rigidity seemed to be lessened more than tremor, but the effect was not consistent. The patients' hemiparesis present after operation decreased as the convalescence progressed.

Other surgeons have removed both areas 4 and 6 and even with this extensive ablation, have not always achieved the desired result.

The results of the operations on the basal ganglia originally carried out by Meyers, according to a review by Browder (1947), have been varied. Of 12 cases operated upon, six were somewhat improved, one had a return of tremor in three years and three died. In a recent report Browder (1948) stated that in his series of 15 cases having a capsular operation, the tremor was abolished in nine cases, ameliorated in three and three patients in whom rigidity was the most prominent feature, died as the result of the surgical procedure.

Cerebral pedunculotomies have been made on seven patients suffering from Parkinson's syndrome. In two cases the tremor returned within a few months (Guiot and Pecker, 1949). In two others there was some return of tremor in one year, and in two no tremor after two years but these latter two patients had a marked hemiparesis.

Spinal pyramidotomy has been more extensively used than any other procedure for the relief of tremor. Putnam and Herz (1950) found in their series of 22 cases followed from 12 to 53 months, that the tremor was abolished in seven and decreased in eight cases. A moderate or more severe paralysis was present in nine patients. In Mashansky's series of 17 cases

treated by anterolateral cordotomy, there were seven failures. Neimeyer (1945) who followed Putnam's technique reported six of his nine cases free of tremor, but the longest follow-up was just one year. Oliver (1949 and 1950) in a series of papers has reviewed 79 cases of unilateral Parkinson's disease in which he had sectioned the lateral columns at the second cervical segment. His best results were in those cases in which the cut was 5 mm. deep and had produced a contralateral sensory loss; 17 of 18 patients having this type of operation were pleased with the result. As the hemiplegia, which was a constant sequelae of the procedure, disappeared in a few weeks, some tremor returned. Ebin (1949) in 11 cases incised through the cervical spinal cord to cut the ipsilateral pyramidal tract and contralateral anterior column. Both tremor and rigidity were much improved by this procedure.

Unfortunately, the results of the various surgical procedures are in most instances based upon a short term follow-up. Whether the end result will be worth-while cannot be stated with certainty at this time. Even a temporary amelioration of a chronic condition might seem a blessing to a patient or relatives. Perhaps, as appears to be true in some cases, medical management may be more effective after an operation. Not until all these factors are known can a final assessment be made.

It must be admitted that the surgery of involuntary movements has not yet become a standardized procedure. The outcome is unpredictable even in the hands of the most experienced operators. The occasional excellent clinical result raises the hope that a technique may be developed which will abolish the involuntary movements without paralysis. But before this can be accomplished we must learn more of the pathogenesis and pathophysiology of the involuntary movements. It may be that when these data are known, we will have chemotherapeutic agents at our disposal which, by selective inhibition of nerve fibers or cells, may correct the disordered neuromuscular mechanisms. But until that time the surgeon has a valuable role to play in the alleviation of involuntary movements.

REFERENCES

Adrian, E. D.: Double representation of the feet in the sensory cortex of the cat. *J. Physiol.*, 98:16P-18P, 1940.

Alajouanine, T., J. Le Beau, and R. Houdart: Résection corticale de la zone motrice (aires 4 et 6) dans un cas d'athétose localisée au bras gauche. *Rev. Neurol.*, Paris, 79:134–137, 1947.

Alajouanine, T., J. Le Beau, and R. Houdart: Résection corticale de la zone motrice (aires 4 et 6) dans un cas d'hémiparkinson gauche. *Rev. Neurol*, Paris, 79:137–138, 1947.

Alpers, B. J., and R. Jaeger: Hemiballism and its control by ablation of the motor cortex. *Tr. Phila. Neurol. Soc., Arch. Neurol. and Psychiat.*, 64:285–287, 1950.

Anschütz: Hirnoperation bei Hemiathetosis. *Berl. klin. Wchnschr.*, 47(2): 1687, 1910.

Bonin, G. von: Architecture of the precentral motor cortex and some adjacent areas. In Bucy, P. C. The Precentral Motor Cortex. Urbana, Ill., University of Illinois Press, 2:7–82, 1949.

Bonin, G. von and Bailey, P.: The Neocortex of Macaca Mulatta. Urbana, Ill., University of Illinois Press, 1947, 164 pp.

Brodmann, K.: Vergleichende Lokalisationslehre der Grosshirnrinde in ihrer Prinzipien dargestellt auf Grund des Zellenbaues. *Leipzig, J. A. Barth*, xii + 324 pp., 1909.

Browder, J.: Parkinsonsim—Is it a Surgical Problem? *New York State J. Med.*, 47:2589–2592, 1947.

Browder, J.: Section of the fibers of the anterior limb of the internal capsule in parkinsonism. *Am. J. Surg.*, 75:264–268, 1948.

Bucy, P. C.: Cortical extirpation in the treatment of involuntary movements. *A. Research Nerv. and Ment. Dis., Proc.*, 21:551–595, 1942.

Bucy, P. C.: The neural mechanisms of athetosis and tremor. *J. Neuropath. and Exper. Neurol.*, 1:224–239, 1942.

Bucy, P. C.: Cortical extirpation in the treatment of involuntary movements. *Am. J. Surg.*, 75:257–263, 1947.

Bucy, P. C.: The surgical treatment of abnormal involuntary movements. *Surg., Gynec. and Obst.*, 90:376–379, 1950.

Bucy, P. C., and D. N. Buchanan: Athetosis. *Brain*, 55:479–492, 1932.

Bucy, P. C., and T. J. Case: Athetosis. II. Surgical treatment of unilateral athetosis. *Arch. Neurol. and Psychiat.*, 37:983–1020, 1937.

Campbell, A. W.: Histological Studies of the Localization of Cerebral Function. Cambridge, University Press, xix + 360 pp., 1905.

Cobb, S., J. L. Pool, J. Scarff, R. S. Schwab, A. E. Walker, and J. C. White: Section of U fibers of motor cortex in cases of paralysis agitans (Parkinson's disease). *Arch. Neurol. and Psychiat.*, 64:57–59, 1950.

Davison, C., and S. P. Goodhart: Dystonia musculorum deformans: a clinico-pathological study. *Arch. Neurol. and Psychiat.*, 39:939–972, 1938.

DeLisi, L., L. Perria, and U. Sacchi: Contributi alla terapia chirurgica delle ipercinesia. II. Tremore post-emiplegico ed attività soppressoria corticale. *Sistema Nervoso*, 1:43–48, 1949.

Delmas-Marsalet, P., and L. Van Bogaert: Sur un cas de myoclonies rhythmiques continuées déterminées par un intervention chirurgicale sur le tronc cérébral. *Rev. Neurol.*, 64:728–740, 1935.

Dusser de Barenne, J. G., H. W. Garol, and W. S. McCulloch: Functional organization of sensory and adjacent cortex of the monkey. *J. Neurophysiol.*, 4:324–330, 1941.

Dusser de Barenne, J. G., H. W. Garol, and W. S. McCullouch: Physiological neuronography of the cortico-striatal conections. *A. Research Nerv. and Ment. Dis., Proc.*, 21:246–266, 1941.

Ebin, J.: Combined lateral and ventral pyramidotomy in treatment of paralysis agitans. *Arch. Neurol. and Psychiat.*, 62:24–47, 1949.

Economo, C. von, and G. N. Koskinas: Die Cytoarchitektonik der Hirnrinde des erwachsenen Menchen. Wien und Berlin, J. Springer, xxxix + 810 pp., 1925.

Ferrier, D.: The functions of the brain. London, Smith, Elder & Co., xv + 323 pp., 1876.

Foerster, O.: Zur Analyse und Pathophysiologie der striären Bewegungstörungen. *Ztschr. f. d. ges. Neurol. u. Psychiat.*, 73: 1–169, 1921.

Foerster, O.: Schlaffe und spastische Lahmung, in Bethe, A. et al. *Handbuch der normalen und pathologischen Physiologie*, Berlin, Julius Springer, 10:922, 1929.

Foerster, O.: The cerebral cortex in man. *Lancet*, 2:309–312, 1931.

Foerster, O., and O. Gagel: Die Vorderseitenstrandurchschneidung beim Menschen. *Ztschr. f. d. ges. Neurol.*, 138:1–92, 1932.

Frazier, C.: Surgery of the Spinal Cord. New York, D. Appleton & Co., 668, 1918.

Gardner, W. J., and G. H. Williams, Jr.: Interruption of the sympathetic nerve supply to the brain—effect on Parkinson's syndrome. *Arch. Neurol. and Psychiat.*, 61:413–421, 1949.

Greenwood, J.: The mechanisms of the corpus striatum and the cerebellum in muscular action. The mechanism of voluntary muscular action. *South. M. J.*, 28:653–659, 1935.

Guiot, G., and J. Pecker: Traitement du tremblement parkinsonien par la pyramidotomie pedonculaire. *Semaine d. hôp. de Paris*, 25:2620–2624, 1949.

Guiot, G., and J. Pecker: Tractotomie mésencéphalique antérieure pour tremblement parkinsonien. *Rev. Neurol.*, 81:387–388, 1949.

Hamby, W. B.: Discussion of paper by Browder, J. Parkinsonism—Is it a surgical problem? *New York State J. Med.*, 47:2592, 1947.

Hines, M.: The "motor" cortex. *Bull. Johns Hopkins Hosp.*, 40:313, 1937.

Horsley, V.: The Linacre Lecture on The Function of the So-Called Motor Area of the Brain. *Brit. M. J.*, 2:125–132, 1909.

Klemme, R. M.: Surgical treatment of dystonia with report of one hundred cases. *A. Research Nerv. and Ment. Dis., Proc.*, 21:598–601, 1942.

Lashley, K. S., and G. Clark: The cytoarchitecture of the cerebral cortex of Ateles: a critical examination of architectonic studies. *J. Comp. Neurol.*, 85:223–305, 1946.

Mashansky, F. I.: Traitement chirurgical des movements involuntaires des extremities appliqué au parkinsonisme post-encéphalitique. *J. de Chir.*, 46:877–899, 1935.

Mettler, F. A., H. W. Ades, E. Lipman, and E. A. Culler: The extrapyramidal system. An experimental demonstration of function. *Arch. Neurol. and Psychiot.*, Chicago, 41:948–995, 1939.

Meyers, R.: The modification of alternating tremors, rigidity and festination by surgery of the basal ganglia. *A. Research Nerv. and Ment. Dis., Proc.*, 21:602–665, 1942.

Meyers, R.: Surgical interruption of the pallidofugal fibers. Its effect on the syndrome of paralysis agitans and technical considerations in its application. *New York State J. Med.*, 42:317–325, 1942.

Meyers, R.: The present status of surgical procedures directed against the extrapyramidal disease. *New York State J. Med.*, 42:535–543, 1942.

Meyers, R., D. B. Sweeney, and J. T. Schwidde: Hemiballismus: aetiology and surgical treatment. *J. Neurol., Neurosurg. and Psychiat.*, N. S. 13: 115–126, 1950.

Money, R. A.: Discussion of paper by W. L. Reid. Studies on the tremor-rigidity syndrome. I. Surgical treatment of human subjects. *M. J. Australia*, 2:532–533, 1948.

Naffziger, H. C.: Discussion of paper by P. C. Bucy, and T. J. Case. Athetosis. II. Surgical treatment of unilateral athetosis. *Arch. Neurol. and Psychiat.*, 37:1017–1018, 1937.

Nasaroff, N. N.: Uber Alkolinjektionen in die kortikale Hirnsubstanz bei Athetose. *Zentralbl. f. Chir.*, 54:1478–1481, 1927.

Negrin, J.: Choreoathetosis in a case of brain tumor with surgical treatment. *Proc. New York Neurol. Soc. J. Nerv. and Ment. Dis.*, 110:173–174, 1949.

Niemeyer, P.: Tratamento cirurgico do tremor parkinsoniano, da atetose e outras hipercinesias. (14 casos operados). *Med. cir. farm.*, 116:654–669, 1945.

Oldberg, E.: Discussion of paper by T. J. Putnam. Results of treatment of athetosis by section of extrapyramidal tracts in the spinal cord. *Arch. Neurol. and Psychiat.*, 39:272–273, 1938.

Oliver, L. C.: Surgery in Parkinson's disease. Division of lateral pyramidal tract for tremor. Report on 48 operations. *Lancet*, 1:910–913, 1949.

Oliver, L. C.: Surgery in Parkinson's disease: complete section of the lateral column of the spinal cord for tremor. *Lancet*, 1:847–848, 1950.

Oliver, L. C.: The present position of surgery in the treatment of Parkinsonism. *Practitioner*, 163:541–543, 1949.

Paillas, J. E., J. Doudouresques, J. Cain, and J. Temalet: Hémimyoclonies datant de 30 ans avec hémiplegie posttraumatiques. Sédation après opération de Bucy. *Rev. Neurol.*, Paris, 70:139, 1947.

Payr, . . .: Athetose. *Münch. med. Wchnschr.*, 68(2):1570, 1921.

Penfield, W., and T. Rasmussen: The cerebral cortex of man. A clinical study of localization of function. New York, The Macmillan Co., xv +248 pp., 1950.

Polenow, A. L.: Ein neuer Weg zur operativen Behandlung einigen Formen von Hyperkinese. *Sovrem. Chir.*, 3:954 (abst. in *Zentralbl. f. Chir.* 56:1147–1148, 1929.

Pollock, L. J., and L. Davis: Muscle tone in Parkinsonian states. *Arch. Neurol. and Psychiat.*, 23:303–319, 1930.

Putnam, T. J.: Treatment of athetosis and dystonia by section of extrapyramidal motor tracts. *Arch. Neurol and Psychiat.*, 29:504–521, 1933.

Putnam, T. J.: Results of treatment of athetosis by section of extrapyramidal tracts in the spinal cord. *Arch. Neurol. and Psychiat.*, 39:258–275, 1938.

Putnam, T. J.: Treatment of unilateral paralysis agitans by section of the lateral pyramidal tract. *Arch. Neurol. and Psychiat.*, 44:950–976, 1940.

Putnam, T. J.: The operative treatment of diseases characterized by involuntary movements (tremor, athetosis). *A. Research Nerv. and Ment. Dis., Proc.*, 21:666–696, 1942.

Putnam, T. J., and E. Herz: Results of spinal pyramidotomy in the treatment of the Parkinsonian syndrome. *Arch. Neurol. and Psychiat.*, 63: 357–366, 1950.

Puusepp, L.: Cordotomia posterior lateralis (fasc. burdachi) on account of trembling and hypertonia of the muscles in the hand. *Fol. Neuropath. Estoniana*, 10:62, 1930.

Rao, L., and R. Morea: Suggestión de tratamiento de los síndromes parkinsonianos por la lobotomiá prefontal (nota previa). *Semana med.*, 56: 133–134, 1949.

Reid, W. L.: Studies on the tremor-rigidity syndrome: I. Surgical treatment of human subjects. *M. J. Australia*, 2:481–492, 1948.

Rizzatti, E., and G. Moreno: Cordotomia laterale posteriore nella cura delle ipertonie extrapiramidali postencefalitiche. *Schizofrenie*, 5:117–122, 1936.

Sachs, E.: The subpial resection of the cortex in the treatment of Jacksonian epilepsy (Horsley operation) with observations on areas 4 and 6. *Brain*, 58:492–503, 1935.

Spiegel, E. A., and H. T. Wycis: Pallidothalamotomy in chorea. *Tr. Phila. Neurol. Soc., Arch. Neurol. and Psychiat.*, 64:295–296, 1950.

Spiegel, E. A., and H. T. Wycis: Effect of thalamic and pallidal lesions upon involuntary movements in choreoathetosis. *Tr. Am. Neurol. Assoc.*, 75:234–237, 1950.

Spiller, W. G., C. H. Frazier, and J. J. A. Van Kaathoven: Treatment of selected cases of cerebral, spinal and peripheral nerve palsies and athetosis by nerve transplantation. With the report of a case of athetosis benefited by operation. *Am. J. M. Sc.*, 131:430–456, 1906.

Thiebaut, F., F. Wolinetz, and G. Guiot: Syndrome du noyau rouge, avec tremblement du type de Benedikt traité par excision corticale. *Rev. Neurol.*, 78:612, 1946.

Tower, S. S.: Extrapyramidal action from the cat's cerebral cortex: motor and inhibitory. *Brain*, 59:408–444, 1936.

Verbiest, H.: Neurosurgical experiences in the treatment of spasmodic torticollis, athetosis and parkinsonian tremor. *Folia Psychiat., Neurol. et Neurochir. Neerl.*, 52:204–226, 1949.

Vogt, C., and O. Vogt: Allgemeinere Ergebnisse unserer Hirnforschung. *J. Psychol. Neurol.*, 25:221–462, 1919.

Walker, A. E.: Cerebral pedunculotomy for the relief of involuntary movements: hemiballismus. *Acta Psychiat. et Neurol.*, 24:723–729, 1949.

Walker, A. E.: Personal observation.

Walshe, F. M. R.: Oliver-Sharpen Lectures on The Physiological Analysis of Some Clinically Observed Disorders of Movement. *Lancet*, 1:963–968, 1024–1028, 1929.

Wilson, S. A. K.: Modern problems in neurology. New York, William Wood & Co., viii + 364 pp.

Winslow, R., and I. J. Spear: Section of posterior spinal nerve roots for relief of gastric crises and athetoid and choreiform movements. *J.A.M.A.* 58:238, 1912.

Woolsey, C. N.: Patterns of sensory representation in the cerebral cortex. *Federation Proc.*, 6:437–441, 1947.

WELL BABY CARE*

F. Howell Wright†

The pleasure and honor which accompanies my invitation to participate in this post-graduate course is tempered by the realization that I shall be expected to maintain the standards set in previous years by my colleague, Dr. Douglas Buchanan. Suspecting that I may fall short of this objective, I should like to begin by establishing an alibi. The disorders which Dr. Buchanan so ably discusses are for the most part universal, definite and based upon demonstrable anatomic changes. Brain tumors are the same in Texas and in Chicago. Some of the topics which I have selected are controversial. They are influenced by factors such as climate, urbanization, economic status, intelligence and the general philosophy of the parents. I recognize that, in my ignorance of the Texas way of life, some of my views may be inappropriate for the group of people among whom you work. Yet I suspect that we shall find a common core of problems and principles which are extant in any group of children.

During the past half century we have seen the practice of pediatrics revolutionized by advances in the fields of bacteriology, biochemistry and food technology. For all but a few of the infectious diseases we now have effective antibiotic or prophylactic weapons. Among child populations which can enjoy the optimal effects of modern medicine, the old-time scourges of infancy have practically disappeared. The children whom we see at the University of Chicago Clinics are such a group, coming mainly from families of high intelligence and good economic circumstances, and living in an area which is protected by a very active and vigilant Health Department. Within this clientele we rarely see diseases such as diarrhea, congenital syphilis, diphtheria, rickets, scurvy, pertussis or important degrees of malnutrition. Doctors working in such a setting find that time released by the abolition of such diseases is immediately commandeered by parents seeking advice about problems

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†Professor and Chairman of Department of Pediatrics.

of development and behavior among their relatively normal children. This is the background from which my experience and opinions are drawn.

The great advances in the control of physical disease were made possible by painstaking experimentation in the fields of biochemistry and bacteriology. As soon as the fruits of such scientific study became evident, it was only natural that similar methods should be applied to the study of infant growth, development and behavior. Intensive studies were undertaken to define accurately the requirements for calories, protein, minerals and vitamins. Similar methods were used to measure the rate at which infants grow, the intervals between feedings, the sleep requirements, stool habits, and the times at which an infant should sit up, walk, talk, learn to use the toilet, and give up various infantile pleasures. The information collected by these legitimate studies was rather widely misinterpreted. A vogue for simplified accuracy lead to an intense concentration upon the *average* values so discovered. Too often these averages were glibly translated into an undeviating expression of the normal, forgetting that they were merely the midpoints in a wide range of values found among normal infants. As a consequence, there arose about twenty years ago, a great fad for raising infants scientifically. Parents expected their doctors or books to provide accurate instructions about the normal requirements and performance of their children during every month of their early lives. Many authorities responded with elaborately detailed instructions telling exactly when to feed, bathe, exercise, and bed the child; specific quantities of milk, barley water, and spinach were prescribed; and disciplinary schemes for instilling good habits were worked out. Throughout, the emphasis was upon making the child conform to the normal standards which had been discovered. A mother who failed to force her child into line was made to feel either that she was an incompetent mother or that her infant was abnormal or bad.

About ten years ago, while conducting our well-baby clinics along such traditional lines, we gradually became aware of a disturbing phenomenon. Many of the mothers whose infants appeared to be thriving and well adjusted, were giving only

lip service to our carefully prepared instructions. Although they could accurately quote the instructions issued at a previous visit, close questioning revealed that they were using them only as a very rough guide. Soon we began experimenting by letting down the bars and permitting some deviation from the letter of our routine. As we found some of our problems decreasing we experimented further until we became thoroughly convinced that individual behavior and needs were of great importance in producing satisified infants and mothers. The sheets of carefully planned instructions were destroyed and we started off on an entirely different tack.

Our current practices of instruction are based upon the idea that all infants are different, right from the start, and hence will require individual handling. The mother is told that her own infant's needs cannot be predicted exactly and that she must become a student of his behavior, learning how to recognize his wants. She is warned that this will often require some trial-and-error experimenting on her part, particularly in the beginning and that it will undoubtedly take a little time before she becomes skilled in meeting his needs accurately. She is not left completely at sea, for a broad description of the variations in behavior of small infants is given, and she is encouraged to consult her doctor if she feels that her child's behavior is too far out of line. Instead of issuing orders to the mother, the doctor serves more as a judge of the infant's progress. He interprets behavior for the mother, suggests occasional modifications, predicts coming developments, and above all reassures the mother about her unnecessary concerns. The net result is to make the mother more self-sufficient and less dependent upon the doctor. It also gives her a wholesome respect for her child as an individual, a respect which will be of great value in determining her approach to problems of management later on in his life. Let us see how this technique works with some of the aspects of infant care.

When she leaves the Lying-in Hospital, the mother is instructed to feed her infant as often as he appears to be hungry. She is told that after a preliminary period of adjustment, her infant will probably want to feed between five and eight times a day. She is advised to call her doctor if he regularly requires

ten or more feedings per day following the first week. No restrictions on quantity of milk are imposed. Mothers who are breast-feeding never know how much the infant receives. The danger of overfeeding at the breast is extremely remote. Mothers who are unable to nurse their infants are instructed to offer a simple unsweetened mixture of evaporated milk and water in a concentration of 40 or 50 per cent. They are told that the infant's daily consumption of formula will probably fall between 18 and 35 ounces per day. Consultation with the doctor is advised if the infant seems unwell or fails to keep within these limits. Actually we have seen a few infants consume more than 40 ounces per day of this formula without harming themselves in any discernible way. The same principles may be followed with other formulas although it is probably wise to restrict the total consumption more closely when food with a high carbohydrate content is being used. In addition to milk, infants receive a polyvitamin mixture once a day. Water between feedings is left optional with the mother, except in hot weather or during illness when she is encouraged to offer extra fluid.

The chief advantage of this method of feeding lies in the elimination of unnecessary battles between mother and infant. The infant is allowed to follow his natural hunger rhythms which vary significantly from one baby to another. Some of the larger infants will automatically select a schedule which requires feeding at regular four-hour intervals. Others will sleep longer intervals during the day and will concentrate much of their feeding activity into the late afternoon and early evening hours. Some elect to sleep from supper-time until 2 or 3 A.M. instead of from 10 P.M. until dawn. It is surprising how tenaciously these latter babies cling to the early morning feeding even if they are awakened for food in the late evening. Smaller infants commonly adopt a pattern of frequent daytime feeding with a longer sleeping interval at night. If the mother is prepared to accept such deviations from the schedules printed in books, she profits by feeding her infant when his appetite is best rather than when he is sleepy or upset from a prolonged bout of crying.

Another advantage of the method lies in the fact that the infant is permitted to declare his individual food requirements. Not all babies grow at the same rate. Some start life as small infants, but are destined to be large children. A growth impulse which pushes them rapidly toward their ultimate size requires a large food intake in order to provide the necessary energy. The average infant will double his birth weight in 5–6 months by consuming 55–60 calories per pound. Recently we watched a ravenous infant who would not be satisfied until he was given 110 calories per pound. The reason behind his unusual appetite became apparent as he changed from a 5 lb. and 4 oz. newborn into a large baby of 20 lbs. by the age of six months—almost a four-fold increase in his birth weight. In contrast, infants of diabetic mothers are frequently heavy at birth. Some of them grow quite slowly during the first few months because they are ultimately going to be small. Their food requirement under these circumstances may be very modest.

Differences in the amount of physical activity also affect food requirements. The small, hyperactive, "colicky" infant uses a good deal of muscular energy during his state of frequent wakefulness. He requires more food per unit of his body weight than does the placid infant who lies or sleeps quietly all day long.

When a mother is following her child's appetite, she learns immediately of changes in his feeding rhythm which occur with increasing age. Since feeding and sleeping are reciprocal activities in the infant, she simultaneously learns his sleep requirements and can arrange for his periods of quiet accordingly. If she learns how to recognize his symptoms of fatigue, she will be able later on to provide suitable naps and bedtimes and avoid the useless attempt to legislate sleep.

The free-feeding technique works best with the relaxed, natural mother who finds that it permits her to follow her maternal instincts in dealing with the infant. Such mothers require almost no supervision. Difficulties arise, however, which must be ironed out. Some mothers with precise preconceived notions about infant care find it difficult to accede to demands which do not conform to standards which they hold precious.

Often some quiet persuasion will help them to do a little experimenting which results in the discovery that too much rigidity in management does not work as well as a more casual approach. Other mothers become so impressed with the desirability of meeting the infant's requests that they carry free-feeding to an extreme, interpreting every signal of discomfort as hunger, and filling the infant's mouth with food nearly every time it is opened. Such mothers obviously need to explore other explanations for discomfort such as temperature, positioning, soiled diapers, noise, or the need for more cuddling or sucking activity. In small, hyperactive, colicky babies who are bottle fed, confusion between hunger and sucking desire is frequent. The mother who sees her infant sucking avidly upon a bottle which he has just emptied and crying vigorously when it is removed, naturally concludes that he is still hungry. If she gives him unlimited quantities of formula he may overload his stomach and regurgitate the excess. The result is messy, but no serious harm results. Eventually the mother will learn to stop short of his maximum capacity and to provide additional sucking activity by reducing the rate of flow through the nipple or by leaving the nipple as a pacifier for him to suck upon when the feeding is complete. Many infants of course learn how to take care of their sucking needs by getting fingers into their mouths. The mother who starts out with the understanding that sucking is a legitimate activity on the infant's part, is often saved from unwarranted and futile attempts to stop thumb-sucking later on.

The free-feeding technique carries a potential long-range danger which must be anticipated by the physician. Some mothers will subscribe so completely to the philosophy of catering to the infant's every whim, that they passively permit him to tyrannize the whole household as he grows. They must be shown that their guiding role changes as the infant matures. Although their first objective is to provide a feeling of security to the newborn child and to meet his instinctual needs, later on they must also prepare him to deal with the inevitable disappointments of life and to learn how to compromise his desires with those of other persons about him. If he encounters no restrictions or direction from his family, he

will find the play yard, the school and the community unhappy and unsympathetic places. The guiding physician must help the mother to apply restrictions gradually, gently, but consistently and above all in a fair manner suited to the child's level of development.

The beginning of the second half of the first year is a relatively tranquil period for most mothers. The early problems have been worked out and the mother is over many of her initial doubts. Greater skill and efficiency in predicting and meeting the infant's needs permit her more leisure time for relaxation and rest. The infant himself is usually tractable, easily satisfied and regards the necessary procedures of the day as pleasant games. The mother now has confidence in her ability to take care of her baby. After appropriate compliments, we generally seize this opportunity to anticipate some of the practical problems which will arise as the infant's development gradually transforms him from a sessile, easily corralled plaything into a crawling, walking, climbing, exploring child with ideas and a will of his own. The manner in which this is done is varied somewhat depending upon the personality of the mother.

The mother who has demonstrated a natural, instinctive facility in meeting her child's changing needs, requires little attention. She is encouraged to give her child opportunity to acquire skill in locomotion and manipulation. Some of the common household dangers are pointed out to her and practical hints are given to help her keep his inquisitiveness within bounds. A mother who is sympathetic to her child's normal developmental needs can be expected to solve most of these problems by herself.

More time must be spent with the mother who has revealed excessively high standards of cleanliness, neatness and orderliness; or who has been impatient with the normal messiness which goes with diapers and early attempts at self-feeding; or who is constantly trying to regulate her child. Such mothers usually have unrealistic ideals which they expect their infants to meet—ideals which are far in advance of the performance which can reasonably be expected for the chronologic age.

They have a tendency to interpret all behavior in adult terms. New achievements in crawling, walking, talking, manipulation, and exploration are enthusiastically welcomed so long as they conform to "good" adult behavior, but are immediately viewed with alarm when they drift into the category of "bad" adult behavior. For example, the infant is supposed to realize that a spoon should be used only for the purpose of putting food into his mouth, and that it should not be used to transfer his meal from the plate to the floor or into his hair. Much time is spent with such a mother in an attempt to anticipate the infant's next steps in development and to correct her notions of what to expect from him. If her excessive expectations are not modified, she may so restrict her infant that he becomes inhibited about trying anything new; or, if her infant is more aggressive and spirited, she may soon find herself in an exhausting struggle to make him conform. Actually, mothers of this sort usually accept the doctor's suggestions with good grace. Most of them are motivated by the fear that they will not, or cannot raise their infants properly and, to avoid criticism, set unreasonable standards to be met. When they find that the doctor does not expect them to make their infants measure up to these artificial standards, they are usually relieved and better able to tolerate normal infant behavior. Unfortunately, this type of mother is often unimaginative and must have specific advice about many details of the child's behavior because she is unable to grasp the general principles involved.

Another type of mother who requires special attention, is the overly permissive mother who hesitates to impose any restrictions upon her child. As mentioned previously, this attitude may stem from a misinterpretation of the free-feeding system used in early infancy. More often, it is based upon fear of losing the child's love. The mother assumes that if the infant is crossed in his wishes, he will not only be temporarily angry at her, but may permanently harbor resentment. Mothers in whom this basic attitude has been revealed do not need to be encouraged to give their infants leeway for physical activity and exploration. Rather they need to be encouraged

to make some legitimate restrictions. The need for restrictions is first discussed in terms of the infant's safety. It is relatively easy to convince the most permissive mother that her child must be prevented from asphyxiating himself by turning on gas stove burners, from electrocution by putting hair pins in base plugs, or from traffic accidents by wandering into the street. Having established the obvious need for such prohibitions, the physician can then proceed to explain that the child who is never restricted in his innocently destructive exploits will be an unwelcome visitor in other homes. Later on his more strenuous aggressions must be discouraged and some co-operation in social dealings must be encouraged if he is to be prepared for the larger experiences of playing with others and in groups.

Toward the end of the second year mothers must be prepared to endure the negativistic period. This arises when the child begins to discover himself as an independent individual. He becomes so charmed with his own ideas that he has little use for the suggestions of others, frequently reaching the position where he cannot reply anything except "No" to any request or suggestion. Mothers who do not understand what is happening often become very much distressed at his apparent complete disobedience and frustrating behavior. In addition to offering an explanation and some reassurance that this is a temporary phase in his development, the physician can also make some simple but effective suggestions. The negativistic child will often co-operate with procedures which are silently carried out when he would strenuously resist the same procedure if it were accompanied by a verbal request or command. If his mother asks or tells him to come and have his clothes changed, he will automatically say "No." He then has an issue to defend. If his mother says nothing and just starts to change his clothes he may not object because no issue has been joined. Another useful suggestion is to let him make his automatic refusal and then wait a few minutes, during which time he may change his mind and be quite willing to go along with that which he has just refused. When the negativistic streak is mild, it can be very amusing; when it is strong, it may be very frustrating to both mother and child.

During the same period in which the mother is learning how to cope with the problems of increasing range of activity, the necessity for restrictions, and the phase of negativism, she also feels under some obligation to do something about toilet training. Unless sensibly handled, this procedure may become a serious bone of contention between mother and child. Many new mothers attack the problem of toilet training with a sense of urgency. They are impatient to eliminate the nuisance of diapers and to assure themselves that their child will conform to modern sanitary standards. Experience during the last war indicated that the methods in vogue a generation ago were not entirely successful, for there was a high incidence of enuresis among draftees. Modern students of personality development are in general agreement that too early and too vigorous attempts at training not only defeat their own ends but in addition have unwholesome effects upon the child's general emotional development.

As in everything else, an individual approach is needed. The mother is encouraged to go slowly and await some indication from the child that he is ready for training. It is pointed out that under any system, few children will be really trained before 14 months of age and many will still be wetting at 36 months. If, during the latter portion of the first year, the mother finds that her infant's stool habits are predictable within a few minutes, no objection is raised if she wishes to place the child upon a potty and catch the stool. She is advised to stop if she runs into objection from the baby or finds that the manoeuvre is not regularly successful. She is also warned that much of what she is doing is training herself rather than the infant and that her scheme may deteriorate if the child's bowel habits change with age. Occasional potting for passing of urine is also permitted if it meets with no objection from the infant and is fairly successful. Some mothers succeed (usually with girls) in training their infants pleasantly and without fuss early during the second year of life.

Considerable time is spent in discouraging mothers from making fruitless, ritualistic attempts at potting the child who is resistive or totally disinterested. When the child is walking and

beginning to learn the significance of the various areas of the house, the regular use of the bathroom as a place to change diapers is encouraged. Praise rather than censure should be given to the child who informs his mother *after* he is wet or soiled. This represents his first stage in forming a conscience about his soiling and should be supported since he will eventually give earlier warning which will permit timely action by the mother. If he is regularly criticized for noting that he is wet, he will begin to conceal or ignore the fact and lose his rudimentary interest in training. For the young child beginning to walk, an open-door policy for the bathroom is probably desirable in order that he may observe how others use the toilet and give him some basis for a desire to imitate them. Later on, as he approaches the kindergarten age, the notion of privacy for himself and the other members of the family should be encouraged.

Not infrequently we see mothers who make no attempt at toilet training whatever, waiting until the child feels that he is being discriminated against in the privilege of using the bathroom. If the mother can restrain her anxiety, this system will almost invariably work sometime between the second and third birthday. Children who train themselves in this fashion usually complete the process within a week or two. Unfortunately, few mothers can wait calmly for the child to take the initiative. The physician's main function is to learn what the mother is attempting and to discourage attempts which are obviously unsuccessful or which are stimulating resistance on the part of the child.

The mother who is schooled from the start to regard her infant as an individual with his own peculiar traits and requirements, usually accepts reassurance about his aberrations from the average pattern of physical development with good grace. She can appreciate the even temperament of a child who develops leisurely and can recognize the tax in energy and ingenuity which parents of the precocious child must expend to save him from reckless self-injury. If her child is abnormally slow she is better prepared to permit him to move at his own pace and is less likely to subject him to frustrating pressures to force progress beyond his capacity.

Such a mother is more willing to accept reassurance about the non-rachitic bowed legs which are destined to straighten with increasing length. She can view the flat feet of the two year old as a transient stage which will improve as his muscles become stronger. She can wait for him to learn how to correct the inefficiencies in his early gait without wasting money on unnecessary special shoes. And eventually she will be better prepared to find that he does not excel in everything, but has his own special talents in sports, school, the arts, or personal relations.

In the foregoing I have tried to show how some of the policies adopted in the early management of the infant may have far-reaching effects upon his adjustment and acceptance into the family. Are these issues really very important? Can the physician expect to wield any influence upon the mother's attitudes through a short office or clinic visit? I believe the answer to both questions is affirmative.

The factors responsible for our rising incidence of mental disorders are undoubtedly multiple. But most analysts of the situation accord an important role to unwholesome influences upon the developing child chiefly through mishandling or neglect by the parents. Psychiatrists, and the analytic school in particular, believe that the period of infancy is often crucial in determining the ultimate mental health of the individual. Certainly the parents are the ones most able to influence character formation if anyone can do so. Efforts to help them rear happy, secure, well adjusted children are of supreme importance in mental hygiene.

The physician who sees small infants is in a particularly strategic position. The new mother comes to him in confusion and uncertainty. Her immediate concerns are for the physical needs of her child, but she is seeking help and usually accepts it avidly. As he ministers to the physical wants of the child, the physician can simultaneously wield a very important influence upon the mother's developing attitudes toward her infant. If the relation between doctor and parent is a sound one, he will soon be requested to participate in decisions which concern the child's emotional adjustment, his schooling and even

his religious and moral training. The degree to which he is consulted will of course depend upon his willingness to listen to the mother's early anxious queries and his success in solving the physical problems of the infant. If he will spend the time in patient and sympathetic consideration of such problems, the baby doctor can exert a powerful influence upon the emotional climate in which his patients grow.

ISOTOPES AND ISOTOPE-LABELED MATERIALS FOR CANCER RESEARCH: AVAILABILITY AND RECENT USES*

PAUL C. AEBERSOLD†

The rapid progress made during the last decade in the fight against cancer can be largely attributed to the extensive array of powerful tools and methods made available for tackling cancer research problems. The most recent and powerful of these are the now well known tracer atoms or isotopes. The radioactive variety of isotopes have also been uniquely applied as tools of cancer diagnosis and therapy.

Isotopes have been used in investigations on cancer and allied diseases to furnish valuable information on the following: (1) role of essential inorganic and trace elements, (2) metabolic pathways of such important biochemical compounds as amino acids, proteins and hormones, (3) fate and action of important drugs and non-metabolites, (4) mode of action of carcinogens, and (5) biochemical effects of radiation. Also, radioisotopes have been used in a large number of investigations designed toward the ultimate use of the isotope as a diagnostic or therapeutic tool.

In the four and a half years since initiation of the Atomic Energy Commission's distribution program more than 44 per cent of some 17,000 radioisotope shipments made for all fields of study have gone for cancer studies to 175 clinics and research laboratories in the United States. Of these shipments 85 per cent have been used for medical diagnosis or therapy and the remaining 15 per cent have been used in research.

Recently attention was called to another indication of the interested in radioisotopes in cancer research. During the 42nd Annual Meeting of the American Association for Cancer Research, Inc., held in Cleveland, over twenty-five of the papers

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†Chief, Isotopes Division, Oak Ridge Operations, U. S. Atomic Energy Commission, Oak Ridge, Tennessee.

given during the three-day meeting reported on various kinds of isotope investigations.

Radioisotopes may be used either as sources of radiation or as tracer atoms. When used as sources for interstitial and external therapy, reactor-produced radioisotopes offer a wide range in type and energy of radiation. But even more important is the fact that they can be used for internal therapy, that is, a type of therapy which takes advantage of the radiation source being incorporated into a normal body metabolite or some other material introduced directly into the body.

Isotopes, especially the radactive variety, are particularly valuable as tracer atoms. Through the use of isotopic tracers, atoms of elements can often be followed in minute quantities, thousands to millions of times smaller than those detectable by chemical means. More important even than this sensitivity is the specificity of the method. A specific batch of atoms or molecules may be labeled uniquely and traced through a series of chemical or physical reactions. They can be traced independently even in the presence of other atoms or molecules of the same substance, and in spite of multiple reactions with numerous other kinds of atoms and molecules. This permits the ferreting out and untangling of complicated processes that often can be followed in no other way. The tracer technique is therefore particularly useful when studying the complex and dynamic functions of a biological system.

Availability

Radioisotopes of most of the known elements are now available in quantities sufficient for wide-scale use. *(Isotopes—Catalogue and Price List)*. Nearly all the biologically useful radioisotopes including such important ones as radiohydrogen (tritium, H 3), radiocarbon (C 14), radiophosphorus (P 32), radiosulfur (S 35), radiocalcium (Ca 45), radioiodine (I 131), etc., are produced routinely and are available continuously Notable exceptions to the list of available items are the radioisotopes of oxygen and nitrogen and in these two cases concentrated stable isotopes are available which for many tracer purposes serve as well.

As produced in the nuclear reactor, radioisotopes are available in the simplest and most generally adaptable forms, that is, as the element, as a simple salt such as the carbonate or nitrate, or as the oxide. In many instances radioisotope users can conveniently use the materials in the simple chemical forms as shipped. Radioiodine and radiophosphorus, for instance, are often clinically administered as solutions of sodium iodide and disodium hydrogen phosphate which are the chemical forms in which the materials are shipped from the production site.

For most biological studies, however, as well as for certain diagnostic and therapeutic applications, radioisotopes cannot be used in simple inorganic chemical forms but must be incorporated into special complex compounds containing the radioisotope. For example, the element carbon is one of the body's major constituents, although not in its elemental form. It is always found in some complex molecule such as an amino acid or carbohydrate. Radiocarbon, therefore, is only useful in tracing the metabolism of an amino acid if it is first incorporated into the specific amino acid to be studied. In many instances it is also necessary to know the exact location of the radioisotope within the molecule, that is, to know which of the atoms within the molecule is labeled. The radioisotope will serve as a true tracer for some complex compound such as an amino acid only as long as it remains a part of the molecule. Conversely the same tracer can often be used advantageously to identify the degradation products from a labeled compound previously introduced into a biological system.

The increased availability of isotope-labeled compounds, perhaps more than any other single factor accounts for the expanded use of radioisotopes in cancer research during the past year. A cursory review of recent literature bears this out. Although a number of specific compounds will be mentioned in the discussion to follow, this will summarize the current status of labeled compound synthesis and availability. In the future isotope-labeled compounds will become even more important to cancer research and other physiological studies.

Isotope-labeled compounds may be prepared either by chemical or biological synthesis. To date somewhere between 400

and 500 labeled compounds are known to have been synthesized. Of these more than 275 have been synthesized by in preparing the non-radioactive counterpart of the same compound (Crompton and Woodruff). Some complex labeled compounds which cannot be prepared by ordinary chemical procedures may be biologically synthesized by the metabolic processes of animals and plants. Radioisotopes in simple chemical form which have been injected into or fed to an animal may subsequently be extracted from the blood, urine, or tissues as complex organic compounds. Isotope-labeled compounds may similarly be extracted from plants. Approximately 150 different compounds have been prepared in this manner (Woodruff and Fowler).

Most of the compounds which have been prepared thus far have been labeled with radioactive carbon 14 although a number of compounds have also been labeled with the radioisotopes, I 131, S 35, and tritium and with the stable isotope, deuterium. These materials may be classified as (1) low carbon intermediates such as hydrogen cyanide, acetylene, sodium formate and methanol, from which more complex compounds may be prepared, (2) acyclic acids and derivatives such as the acetic acid and succinic acid, (3) amino acids such as alanine and methionine; (4) cyclic compounds such as benzene and phenanthrene, (5) hetrocyclics such as azaguanine and diaminopurine, (6) sugars such as glucose and sucrose, and (7) miscellaneous compounds such as urethan and diiodofluorescein.

Of the large number of isotope-labeled compounds which have been synthesized to date, more than 150 are routinely available either from Commission laboratories or from five private laboratories engaged in the synthesis and distribution of these special materials. *(Isotopics—Announcements of the Isotopes Division).* Each of the specific compounds mentioned above together with other examples of the same categories are now routinely available.

The Commission has also made contract agreements with the five private laboratories now distributing isotope-labeled compounds and five others to develop procedures for the synthesis of an additional 80 labeled compounds. These include compounds which are in demand by numerous investigators but which are

not now routinely available. Items of particular interest to cancer research which fall into this category include the hetrocyclics, folic acid and thiouracil, the steroids, cholesterol and progesterone and the carcinogens, methylcholanthrene and acetylaminofluorene. When satisfactory procedures have been developed for the synthesis of these compounds, they will be made available at prices which do not reflect the cost of development.

Two other aspects of isotope-labeled compound availability which should be of interest to this group are a stockpiling program and a registry program, both of which were recently initiated by the Commission. The stockpiling program involves the purchase by the Commission of certain compounds which research workers have experienced considerable difficulty in obtaining in small quantities at reasonable prices. The Commission will buy a number of such compounds in multimillicurie quantities for stockpiling and will resell them to individual investigators in submillicurie amounts. Compounds of interest to cancer research which have already been included in this program include guanine sulfate, folic acid, thiouracil, testosterone, cholestenone, cholesterol, estrone and tryptophan.

The registry of isotope-labeled compounds is designed to serve isotope users in two ways: (1) to find out if a specific compound is available and from where, and (2) to inform the Commission through the Isotopes Division that they have prepared a specific compound in excess of their needs and are willing to make it available to other users.

By way of a summary statement on availability several points should be noted: (1) radioisotopes of most of the known elements in simple chemical forms as produced in the nuclear reactor are now available in quantities sufficient for qualified investigations and applications in all fields, (2) past experience has demonstrated that the future use of isotopes, especially in the biological fields, will depend to an increasing extent on the availability of a wide variety of isotope-labeled compounds, and (3) the rate at which new labeled compounds become available will be governed in part by the ingenuity and co-operation of investigators requiring them for their research programs.

Research Applications

There are already many proved examples of the value of isotopes to cancer research; some of these will be mentioned. Perhaps the most striking, however, is that through the use of isotopes it has been possible to establish the concept of the "metabolic pool" through which pass most of the body's metabolites. Isotopes not only offer a method for determining what elements and compounds pass through this pool but also the mechanism of their passage.

The large number of body constituents and related substances which have been labeled and traced through various complex body processes include such materials as phospholipids, sugars, proteins, nucleic acids, vitamins, hormones, antibodies, amino acids, drugs, dyes, organic acids, and blood cells. Examples of such studies which are, of course, directly or indirectly related to the study of cancer will be considered in the order mentioned in the introduction.

Radioisotopes have been used in numerous investigations to study the role of essential inorganic and trace elements. For example, Lansing, Rosenthal and Kamen have used radioactive calcium to study calcium ion exchange in normal tissues and in epidermal carcinogenesis. These investigators were able to show through tracer studies that epidermis, unlike other soft tissues, actively exchanges calcium. In early methylcholanthrene-induced hyperplasia there is a loss of calcium storage capacity in epidermis. Thirty day hyperplastic epidermis and a transplantable squamous cell carcinoma were found to be characterized by an inability to exchange calcium ions.

Berlin has similarly used radioactive cobalt to determine the distribution of injected cobalt in the rat. More specifically the distribution was followed during the development of polycythemia in the tissues known to have a hematopoietic function and in the kidneys in an attempt to find some clue to the mechanism of the hematopoietic action of cobalt. Through these tracer studies the quantitative distribution of the cobalt was determined in the liver, spleen, kidney, lung, blood, and bone marrow of the polycythemic rats. It was found that the cobalt rapidly reaches a saturation level in the liver, kidney, and

lungs, and, with a trend toward a slow increase in the red blood cells, in spleen, and bone marrow on prolonged administration.

Representative of a tracer study on the metabolic pathway of an important biochemical compound is the investigation carried out by Griffin, Bloom, Cunningham, Teresi, and Luck on the uptake of labeled glycine by normal and cancerous tissues of the rat. In this particular study glycine labeled with radioactive carbon 14 was administered by stomach tube to rats with hepatomas. The animals were sacrificed at intervals, and the rate of incorporation of the isotope was determined in whole-tissue homogenates and also in the proteins of liver, hepatoma, and other tissues and organs. It was reported that liver tissue and also liver protein showed a maximum incorporation of the radioisotope within two hours, while the tumor tissue did not reach a maximum until twelve hours after administration of the labeled glycine. Nucleoprotein fractions prepared from the nuclei and the cytoplasm of the liver tumors exhibited a delayed uptake of the isotope, as compared to similar fractions obtained from the noncancerous liver. Also, of the tissues studied, liver, kidney, bone, serum, and spleen exhibited the greatest uptake of the isotope while muscle was relatively inactive.

The general distribution of the isotope as found in these studies is essentially in agreement with that reported by other investigators. The findings are, however, in contrast with those of *in vitro* experiments carried out by Zamecnik. Both Griffin and Zameçnik agree that one possible reason for the difference is that in the *in vitro* studies, an excess of the amino acid is always available whereas in the whole animal this may not be the case.

Riegel, Hartop and Kittinger have used the radiotracer technique in determining the metabolism of a steroid hormone, progesterone. The experiments which were a continuation and extension of earlier studies made with mice were carried out in rats with labeled progesterone-21-C 14.

It was found through the tracer studies that progesterone is not appreciably stored in the tissues but is eliminated in the feces and this elimination is nearly complete within 12 hours. Part of the administered progesterone was known to have been

oxidized since radioactive carbon dioxide derived from the side chain was exhaled. Large amounts of radioactivity were found in the liver, and the pituitary and ovaries were also found to contain relatively large concentrations of radioactivity. Radiocarbon has similarly been used by Samuels in studying the *in vitro* metabolism of testosterone in rat liver.

An example of a study which clearly illustrates the specificity of the tracer technique is the work reported by Marshak on the difference in rate of radiophosphorus turnover of different types of nuclei in rabbit liver. He reports that by differential centrifugation it has been possible to separate different types of nuclei from rabbit liver, that is, to separate spherical, elongated and lobulated nuclei. He also noted that the total P 31 content per nucleus is the same for a fraction containing 99 per cent spherical nuclei and 1 per cent elongated nuclei as for a fraction containing 1 per cent spherical nuclei and 99 per cent elongated nuclei. After intravenous P 32 administration, however, the specific activity of the latter fraction is lower than the first by 30 per cent. As the author indicates, this experiment gives an example of a procedure whereby physiological activity of nuclei of tumor and stroma cells may be followed separately.

Although these are but a few examples of the ways in which radioisotopes have been used in metabolism studies they do illustrate the unique usefulness of the tracer technique. The primary importance of isotope utilization in cancer research lies in the fact that they permit detailed observations of the mechanism of various metabolic processes in normal as compared with malignant tissues.

The second major type of isotope investigation is concerned with using a tracer to follow the fate of a drug or some other material not considered a normal body metabolite. For example, Skipper *et al.* have used labeled nitrogen mustard or more specifically methyl bis (2-chloroethyl) amine labeled with radiocarbon 14 in the methyl position to study the mechanism of the anti-leukemic activity of this compound. The experiments described here were designed to determine the fate of the molecule or portion thereof in normal and leukemic mice. Following injection of the labeled nitrogen mustard, the mice

were placed in metabolism chambers and the expired carbon dioxide and urine collected. Subsequently the animals were dissected and the organs and tissues taken for radioactivity determinations.

The investigators reported, as had been expected, that the active carbon from the methyl labeled nitrogen mustard was distributed throughout all organs and tissues. The specific activity of the blood serum was higher than in the cells and the small intestine was consistently the tissue of highest activity. The spleen was found to have fixed carbon from the nitrogen mustard at concentrations five to ten times that of the bone marrow which was one of the lowest activity tissues. Although no marked difference was found in active carbon retention between normal and leukemic mice, the thymus and lymph nodes were somewhat higher in activity in the leukemic groups.

A somewhat similar study has recently been reported by Weaver, Tolbert, and Kreuckel where a tracer was used to determine the influence of tumors on liver concentration of stilbamidine. In this study, a series of normal and tumorous mice were injected with stilbamidine labeled with C 14. After 96 hours, the livers were removed and analyzed for radioactivity. It was found that much higher and distinctly abnormal concentrations of the radioisotope were present in the livers of the tumor bearing mice as compared to the controls for the *A* and *dba* strains. By way of explanation the authors have advanced the possibility that stilbamidine precipitates in the liver cells as an insoluble stilbamidine-nucleate complex and the increased amounts present in tumor bearing animals are related to increased formation of certain nucleic acids in these livers. Further studies are now being conducted to determine the chemical nature of the stilbamidine complex or derivative that concentrates in these livers and in what portions of the liver this material is located.

Through studies of this type the tracer technique has been able to give an insight into the mechanism of the chemotherapeutic effect of a number of compounds.

In the third type of isotope investigation, that is, where the tracer is used to determine the action of injurious agents, this

group will be particularly interested in studies with labeled carcinogens. To date the tracer technique has been used to study the action of such labeled carcinogenic hydrocarbons as methylcholanthrene, dibenzanthracene and acetylaminofluorene.

One such study has been carried out by Morris, Weisburger, and Weisburger with carbon 14 labeled acetylaminofluorene. Earlier workers had found through chemical and colorimetric analyses that only one third of an administered dose of acetylaminofluorene could be accounted for in the tissues and excreta of the experimental animals. This suggested that the acetylaminofluorene had been transformed into metabolites which did not possess the aromatic amino group. It also suggested the possibility that one of these metabolites might function as the primary carcinogen.

In the work described here the metabolic path of the acetylaminofluorene was followed after having been labeled in the 9 position, that is, at a stable point in the molecule which would not be readily broken down during metabolism. Also to investigate the role played by the acetyl group, the distribution of the same carcinogen was determined after having been labeled with radiocarbon in the methyl group of the acetlyamino side chain.

Although only preliminary results of this study have been reported it has been shown that the distribution of radioactivity in the two cases is considerably different. For example, six per cent of the radioactivity from the carcinogen labeled in the side chain was eliminated in the breath within 6 hours after administration whereas a negligible amount of radioactivity from the same compound labeled in the 9 position was expired during the same period. Also the path taken by the radioactivity of the 9-labeled compound was different from that of the methyl-labeled derivative. Less of the methyl-labeled derivative was excreted in the urine, and more was found in the organs and tissues. It was concluded that the acetyl group of 2-acetylaminofluorene is split off by the rat, at least in part, and metabolized independently of the remainder of the molecule.

Effects of Radiation

Also of interest here is the use of radioisotopes to study the effects of radiation. One type of study utilizes the radioisotope to trace the fate of a metabolite or some other material in the body when the whole body or a portion of it is subjected to an external source of radiation. When observations made under these conditions are compared with normal controls, a very clear insight into a variety of radiation effects can be gained.

An investigation of this type has recently been reported by Kelly and Jones. In the investigation, designed specifically to determine the effects of irradiation on nucleic acid formation, the radiation was supplied from an internal rather than an external source, that is, from colloidal yttrium 90 introduced intravenously and intramuscularly into the experimental mice. The experiments were conducted on mice of the *A* strain bearing bilateral transplants of mammary carcinoma using radioactive phosphorus 32 as a tracer for the nucleic acid. Findings were based on specific activity measurements made on desoxypentose nucleic acid isolated from the tumors and the livers of the mice. It was, of course, also necessary to separate the yttrium 90 from the samples before making specific activity determinations of the desoxypentose nucleic acid.

It was shown that the radioactive colloid when injected intravenously localizes in the liver and when injected intramuscularly, remains at the site of injection. The animals whose livers were irradiated showed only 66 per cent of the tumor nucleic acid specific activity of the control group. The animals whose muscles were irradiated showed 84 per cent of the tumor nucleic acid specific activity of the control group. The turnover rate of the desoxypentose nucleic acid was thus shown to be effected in both cases by the radiation.

Still another type of investigation is designed to determine the radiation effects of isotopic materials used in diagnosis and therapy. For example, Warren, MacMillan and Dixon have recently reported on an extensive study of the effects of internal irradiation of mice with radioactive phosphorus. In this investigation P 32 was subcutaneously administered to a large number of mice and subsequent detailed studies made on the

spleen, lymph nodes, thymus, bone, bone marrow, gonads, kidneys, adrenal glands, digestive tract, spinal cord, lungs and liver. The results of their observations were correlated with the amount of radiophosphorus injected and the length of time elapsed between injection and observation.

A similar study has also been reported by Koletsky, Bonte, and Friedell. These workers found that one of the late effects observed in rats injected with large doses of radioactive phosphorus was the development of malignant neoplasms. The radiation doses per gram of body weight were much larger than those used in therapy. Of thirteen tumors produced, ten were osteogenic sarcomas, situated most frequently in the jaw and also occurring in the spine, tibia, femur, or ilium. Four of the neoplasms metastasized to the lungs. There were three squamous cell carcinomas involving the face and associated with exophthalmos. One particularly interesting feature of this study was the speculation that the neoplasm occurs as part of, or as the end result of atypical bony growth representing attempted regeneration in regions of radiation necrosis.

Diagnostic and Therapeutic Applications

The diagnostic applications of radioisotopes fall into two main categories: (1) where the isotope is used to determine the flow or volume of body fluids and (2) where the application depends on the selective absorption or differential turnover of the isotope in a specific tissue.

The first type is of no particular interest to the cancerologist unless made as an adjunct to other methods of diagnosis or possibly during surgery. Techniques included in this classification are determinations of blood plasma volumes and circulating blood volumes, blood circulation to the extremities and radiocardiography. The second type comes much closer to your interests and includes the measurement of radioactive iodine uptake and excretion as a diagnostic test for thyrotoxicosis and the uptake of radioiodine labeled diiodofluorescein for diagnosing and localizing certain types of brain tumors.

A number of groups are currently attempting to find new ways of using radioisotopes for diagnostic purposes. Most of these are tracer studies designed to determine the degree of

concentration of a radiomaterial in a malignant tissue not from the standpoint of determining the physiological activity of the compound as from the standpoint of finding a material which will concentrate sufficiently in a diseased tissue to use its unique rate of uptake or turnover as a diagnostic test. For example, Moore and co-workers who have used radioiodine labeled diiodofluorescein extensively for locating the site of brain tumors have recently reported that iodinated plasma protein is as satisfactory for this particular diagnostic test.

Myers has used radioactive sulfur to study the distribution of 70 different labeled acidic and basic dyes (derivatives of sulfanilic and diiodosulfanilic acids) following injection into cancer-bearing mice. Eighteen of the compounds tested exhibited a selective uptake in tumor tissue by a factor five times greater than the uptake in normal tissue. The most favorable ratios (as high as 20) were usually found in Sarcoma 37 at 96 hours after injection. In general, uptake of a radiomaterial in tumor tissue by a factor of five times its uptake in normal tissue is sufficient for a diagnostic application. Although radioactive sulfur was used as a tracer in these studies, diagnostic applications of the compounds would probably utilize radioactive iodine as the radioactive label, thereby permitting detection of the compound from outside the body.

Ray and Soffer have recently reported on a similar investigation carried out with a series of sulfur 35 labeled sulfonamides. It is interesting to note that these investigators selected this series of compounds because (1) the sulfonamide linkage is apparently not attacked by enzymes, (2) sulfonamides have been shown to reduce the effective vitamin intake of animals by suppressing the intestinal flora, and this reduction of essential vitamins in the diet of tumor-bearing mice is known to inhibit the growth of tumors, and (3) sulfonamides have been reported to localize in certain tissues.

Therapy

The therapeutic uses of radiomaterials may similarly be divided into two categories: (1) where localization of the isotopic material depends on its physical placement in the diseased

tissue and (2) where localization depends on biochemical placement.

Applications of the first type are similar in many ways to applications made of radium and x-rays during the past twenty-five years. One recent development of particular interest reported by Myers is the use of radiocobalt-nylon thread for topical and interstitial therapy of accessible tumors. Still another is the use of multicurie sources of radiocobalt as external irradiators for treating deep-seated bone tumors. Such irradiators would be used in the place of x-ray machines operating in the one to two million volt range and would have several advantages over x-ray machines from the standpoint of radiation characteristics, size and ease of operation. Although several such irradiators have been designed, it is probable that the first will be established through a co-operative program of the M. D. Anderson Hospital here at The University of Texas and the Oak Ridge Institute of Nuclear Studies in Oak Ridge.

The second type of therapeutic application, that is, the type which depends on localization of the radiomaterial by biochemical placement, makes the wider and more unique use of nuclear reactor-produced radioisotopes. The principal difference between the use of an isotope as a diagnostic tool and a therapeutic tool is the larger amount of radiomaterial required and hence the larger selectivity required.

Radioiodine has, of course, been used extensively for the treatment of hyperthyroidism. It has not proved very successful for treating primary cancer of the thyroid although it has proved useful in the treatment of metastases from the primary tumor. As Seidlin has pointed out, "In a patient with primary carcinoma of the thyroid gland and multiple metastases, it is unlikely that either the primary tumor or the metastases will show significant radioiodine uptake unless the primary tumor has destroyed most of the non-neoplastic thyroid gland. If spontaneous uptake does occur, the therapeutic procedure is obvious. If it does not, a complete thyroidectomy, surgical or by means of radioiodine, is indicated. Five or six weeks after the thyroidectomy, most metastases show uptake of radioiodine."

Radioactive phosphorus has been used effectively in treating certain blood dyscrasias such as polycythemia vera and chronic leukemia. This application is not based on a large selective absorption but rather on a combination of more rapid turnover and somewhat greater concentration of phosphorus in the blood producing system. Today radiophosphorus is considered the treatment of choice of many physicians in treating polycythemia vera and has been found to offer some relief in certain cases of chronic leukemia although it is generally considered unsatisfactory for acute leukemia.

Erf has reported that during the decade, 1940 to 1950, over 1,000 cases of polycythemia vera were treated with radiophosphorus. Over 85 per cent of these cases have experienced satisfactory clinical and hematological remissions which lasted from six months to over nine years, so far. In approximately 66 per cent of the cases the remissions lasted about two years. Erf has also reported that 1,000 cases of leukemia were similarly treated with radiophosphorus during the same decade. Although life may not have been prolonged in these cases, the usefulness and comfort of the patients were greatly improved.

Concluding Remarks

A more extensive use of radioisotopes in future cancer research may be expected. Also new and improved methods will be developed for using them in cancer diagnosis and therapy. Of the two, the use of radioisotopes as tracer atoms for ferreting out information on cellular metabolism and other processes will probably prove more valuable in cancer than their use as diagnostic and therapeutic agents.

In any event the rate of growth and expansion in both instances will depend to a large extent on an increased availability of a wider variety of isotope-labeled compounds and on more people trained to handle and use redioisotopes. It will also depend on a wider appreciation of the power and versatility of the radioisotope as a research tool.

REFERENCES

Berlin, Nathaniel I.: "The Distribution of Cobalt in Polycythemic Rats", *J. of Biol. Chem.*, 187, No. 1, 41, 1950.

Crompton, Charles E., and Nathan H. Woodruff: "Chemical Syntheses of Radioisotope-Labeled Compounds", *Nucleonics*, 7, No. 3, 49 and No. 4, 44, 1950.

Erf, L. A.: "Radioactive Isotopes: Some of Their Medical Uses", *Practical Therapeutics*, 1951.

Griffin, A. Clark, Sanford Bloom, Lew Cunningham, Joseph D. Teresi, and J. Murray Luck: "The Uptake of Labeled Glycine by Normal and Cancerous Tissues in the Rat". *Cancer*, 3. No. 2, 316, 1950.

"*Isotopics—Announcements of the Isotopes Division*", available from Isotopes Division, U. S. Atomic Energy Commission, Oak Ridge, Tennessee.

Isotopes—Catalog and Price List, available from Isotopes Division, U. S. Atomic Energy Commission, Oak Ridge, Tennessee.

Kelley, Lola S., and Hardin B. Jones: "Effects of Irradiation on Nucleic Acid Formation." *Proc. Soc. Exper. Biol. and Med.*, 74, 493, 1950.

Koletsky, Simon, Frederick J. Bonte, and Hymer L. Friedell: "Production of Malignant Tumors in Rats with Radioactive Phosphorus." *Cancer Research*, 10, No. 3, 129, 1950.

Lansing, A. I., T. B. Rosenthal, and M. D. Kamen: "Calcium Ion Exchanges in Some Normal Tissues and in Epidermal Carcinogensis." *Archives of Biochemistry*, 19, 177, 1948.

Marshak, Alfred: "Difference in Rate of P 32 Turnover of Different Types of Nuclei in Rabbit Liver." *Cancer Research*, 10, No. 4, 232, 1950.

Moore, G. E.: University of Minnesota, private communication.

Moore, G. E., D. A. Kohl, J. F. Marvin, J. C. Wang, and C. M. Caudill: "Biophysical Studies of Methods Utilizing Fluroescein and Its Derivatives to Diagnose Brain Tumors." *Radiology*, 55, No. 3, 344, 1950.

Morris, Harold P., John H. Weisburger, and Elizabeth Weisburger: "The Distribution of Radioactivity Following the Feeding of Carbon 14-Labeled 2-Acetylaminofluorene to Rats." *Cancer Research*, 10, No. 10, 620, 1950.

Myers, William G.: "Localization of Radioactive Compounds in Tumors." *Cancer Research*, 10, No. 4, 234, 1950.

Myers, William G.: Presented at Thirty-Sixth Annual Meeting of the Radiological Society of North America, Inc., Chicago, Illinois, December, 1950.

Ray, Francis Earl, and Louis Soffer: "Compounds for Cancer Research. V. Radioactive Sulfonamides." *The J. of Organic Chem.*, 15, No. 5, 1037, 1950.

Riegel, Byron, W. L. Hartop, and G. W. Kittinger: "Studies on the Metabolism of Radioprogesterone in Mice and Rats." *Endocrinology*. (To be published.)

Samuels, Leo T.: "Intermediary Metabolism of the Steroids." Fifth Annual Report of the Committee on Growth of the National Research Council to the American Cancer Society, June, 1950, page 177.

Seidlin, S. M.: "Radioiodine as a Diagnostic and Therapeutic Tool in Clinical Medicine." *Recent Progress in Hormone Research*, 4, 483, 1949.

Skipper, Howard E., et al.: "Overall Tracer Studies with C 14-Labeled Nitrogen Mustard (NH2) in Normal and Leukemic Mice." Progress Report on the Chemotherapy of Leukemia and Studies on the Mechanism of Action of Certain Anti-Cancer Agents, Southern Research Institute, Birmingham, Alabama, August 15, 1950.

Warren, Shields, Jane C. MacMillan, and Frank J. Dixon: "Effects of Internal Irradiation of Mice with P 32," Parts I, II. *Radiology*, 55, No. 3, 375 and No. 4, 557, 1950.

Weaver, John C., Bert M. Tolbert, and Barbara J. Kreuckel: "Influence of Tumors on the Liver Concentration of Radioactive Stilbamidine." University of California Radiation Laboratory Report 963, October 23, 1950.

Woodruff, Nathan H., and E. Eugene Fowler: "Status of Biological Syntheses of Radioisotope-Labeled Compounds." *J. of the Tennessee Acad. of Scie.*, 24, No. 4, 229, 1949.

Woodruff, Nathan H., and E. Eugene Fowler: "Biological Syntheses of Radioisotope-Labeled Compounds." *Nucleonics*, 7, No. 2, 26, 1950.

Zamecnik, P. C.: "The Use of Labeled Amino Acids in the Study of the Protein Metabolism of Normal and Malignant Tissues: A Review." *Cancer Research*, 10, No. 11, 659, 1950.

Zamecnik, P. C., I. D. Frantz, Jr., R. B. Loftfield, and M. L. Stephenson: "Incorporation in vitro of Radioactive Carbon from Carboxyl-labeled DL-Alanine and Glycine into Proteins of Normal and Malignant Rat Livers." *J. of Biol. Chem.*, 175, 299, 1948.

THE GENESIS OF BREAST CANCER IN MICE*

JOHN J. BITTNER

Inbred strains of mice have been selected which have different potentialities for the development of various types of neoplasia. By studying stocks with high and low incidences of spontaneous mammary cancer and the hybrids derived by reciprocal crosses, it has been possible to demonstrate several causative factors. In these studies, it was shown that some material influence was involved in the genesis of these tumors (Jackson Memorial Laboratory, Staff of: 1933) and by foster nursing the young born to females of cancerous strains soon after birth, this was demonstrated to be transferred in the milk of females of the cancerous stocks (Bittner, 1936). In 1939 (Bittner) the theory was advanced that mammary cancer in mice generally results from the interaction of three causative factors: the mammary tumor milk agent, inherited susceptibility, and hormonal stimulation.

The mammary tumor milk agent has the properties of an infectious agent or virus since it increases in amount in living cells, remains active following lyophilization, desiccation, filtration, etc., is specifically antigenic, and is macro-molecular in size, etc.

Although the milk agent is involved only in the development of mammary cancer in mice, the agent is distributed throughout the body and no tissue has been tested that did not have the agent. This may be demonstrated by the injection of extracts from either normal or cancerous tissues into suitable

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test animals (susceptible mice without the agent). While the agent could not be recovered from the embryos, it could from the placenta (Dmochowski, 1949).

As shown by serial dilution studies, there may be considerable variation in the activity and/or concentration of the agent in different tissues from the same animal and comparable results may not be obtained when the same tissues from different cancerous hosts of the same stock are assayed. Such studies are complicated by the fact that the experiments may require more than two years to complete and, in some instances, the final observations may be influenced by factors beyond the control of the investigator.

The role of the genetic susceptibility in the development of mammary cancer is not understood and the possibility must be considered that several inherited factors may eventually be demonstrated. Animals of one strain may neither have mammary cancer when they possess the milk agent and are continued as breeders to insure an adequate hormonal stimulation nor will they transfer the agent for more than one generation (Andervont, 1940), whereas mice of another line of the same stock may remain non-cancerous and transmit the agent for several generations (unpublished data). The determination of the number of genes controlling any factor is difficult to ascertain because mice with the same genetic constitution may not have the same incidences when they obtain the "agents" from different cancerous stocks (Bittner, 1948). Furthermore, in one study it was found that the incidence in the progeny was influenced by the age at which the mothers developed cancer and the litter in which the young were born. Linkage has been demonstrated (Bittner, 1944).

The hormones associated with pregnancy and lactation will accelerate the development of mammary cancer. Whereas females of the A strain have a low incidence when they are kept as virgins, the breeders show a high incidence.

When day-old mice of various strains were ovariectomized, Woolley, Fekete, and Little (1939, 1940, 1941) observed that many of the castrated females of the dilute brown stock developed mammary cancer. Postcastrational hyperplasia of the adrenal cortex was found to be the source of the hormonal

stimulation. Similar adrenal changes were noted in ovariectomized females of the C3H strain while members of the C57 black (B) strain showed neither the adrenal response nor stimulation of the secondary sex organs. Adrenal carcinoma developed in castrated mice of the Ce stock (Woolley and Little, 1945).

Since two of the strains that showed postcastrational adrenal hyperplasia gave high incidences of mammary cancer when the females were maintained as virgins, studies were initiated to ascertain if this response might be associated with a high incidence in virgin females.

The hormonal mechanism, related to a high incidence of mammary cancer in virgin females of one cancerous stock and their hybrids, was found to be under genic control, and has been called the inherited hormonal influence (Bittner, 1945). The transmission of the inherited hormonal influence may be demonstrated by mating females of the A strain with males of stocks that transmit the mechanism. While the virgins of the A strain have a low incidence of mammary cancer, F_1 virgins will have a high incidence when the males of the paternal stock carry the hormonal factor; if the hybrids do not receive the inherited hormonal influence from either parent, they give a low incidence (Bittner, 1939).

The relationship between the inherited hormonal influence, postcastrational adrenal hyperplasia, and a high incidence of mammary cancer was established in mice of the Z (C3H) stock and the AZ hybrids while the castrated mice of the A strain showed no evidence of hormonal stimulation (Smith and Bittner, 1945; Smith, 1948). Further studies determined that several stocks transmitted the inherited hormonal influence based upon an incidence of mammary cancer of at leastt 70 per cent in F_1 virgins with mothers of the A strain. These are the Z, D_8, D_2, C, and I strains. Virgins of the C stock have a high incidence when they possess the milk agent (Andervont, 1949) as will those of the Z, D_8, and D_2 stocks. Although the breeders of the I strain will show a low incidence when they have the milk agent (Andervont, 1945), the castrated females gave the characteristic adrenal response (Huseby and Bittner, 1948).

Thus, although mice of the I stock are relatively non-susceptible for the development of spontaneous mammary cancer, mice of this strain will transmit the inherited hormonal influence. This shows that the same genes do not control the inherited susceptibility for mammary cancer and the hormonal mechanism as indicated in previous reports.

When females of the Ce stock obtain the milk agent by foster nursing, the breeders have a high incidence but preliminary data indicate that the virgins have a low incidence of mammary cancer. The $AceF_1$ virgins have an incidence of approximately 37 per cent, considerably lower than one would expect if the Ce mice possessed the inherited hormonal influence. The hormonal pattern found in mice of the NH stock may be still another inherited mechanism for when females of the Z strain are mated with males of the NH stock, the time of appearance of mammary cancer in the $ZNHF_1$ virgins is much later than in crosses between Z females and males of strains with the inherited hormonal influence (unpublished data). The incidence of mammary cancer in the $ANHF_1$ virgins has been found to be 16 per cent and the expected high incidence in F_1 breeders is not being obtained.

The inherited hormonal influence is another cause of mammary cancer in mice and is related to the development of a high incidence in virgin females of inbred stocks or F_1 hybrids which also have the inherited susceptibility and the milk agent. Part of the physiological effects of this mechanism is due to an inherited ovarian function (Bittner and Huseby, 1948 and 1950).

The work at Samuels *et al.* (1947) may offer some information as to the possible role of the milk agent in the genesis of mammary cancer in mice. It was determined that animals with the agent excreted a smaller amount of 17-Ketosteroids than did mice of the same stock without the agent. The injection of an extract with the agent altered the excretion rate to compare with that found in mice of the same stock that had received the agent by nursing. Each strain or group of hybrids showed different rates. Thus, mice of a strain with a low incidence may not be used as controls for animals of another

stock with a high incidence of mammary cancer in these studies.

To produce a high incidence of spontaneous mammary cancer in mice the three causative factors mentioned above must interact. That a few tumors would develop in animals where one of these factors could not be demonstrated, has been obvious for many years. Heston *et al.* (1950) have recently reported that in a subline of mice without the milk agent of the susceptible C3H stock, 38 per cent of the breeders will develop mammary cancer at an average age of 20.3 months as contrasted with an average age of 7.8 months, and an incidence of 97 per cent, in females with the agent. Only 4 per cent of the agent-free females have cancer when they are not permitted to breed. In our subline of the Z or C3H stock without the agent, continued for more than 30 generations, less than 1 per cent of the breeders give rise to cancer (Bittner, 1948).

If the observations made on experimental animals might have any application to the problem in humans, it might be possible to account for the high incidence of breast cancer among unmarried women as resulting, perhaps in part, from the physiological effects of some inherited hormonal pattern. The clinical effects seen following castration in males with prostatic cancer may be due to a comparable factor.

Because of the investigations on mammary cancer in mice, several surveys have been initiated to determine if a nursing influence may be involved in the genesis of breast cancer in humans (perhaps for only one type of breast cancer). It is known that in mice the so-called milk agent may be recovered from the serum and resuspended blood cells of infected animals (Bittner, 1946–47) and may be transferred by the male parent (Andervont and Dunn, 1948). In one study, females of a susceptible stock but without the milk agent were mated to males with the agent and approximately 50 per cent of the females became "infected" and developed mammary cancer. Only a small percentage of the progeny born to these females, in their first five litters later had cancer but nearly 100 per cent of the progeny born to "infected" females in their seventh and following litters died with mammary tumors. By following the progeny, the approximate time of infection of the mothers could

be determined. One noncancerous mother was found to transfer the agent to her progeny. In another cross involving males of the same stock, only 3 per cent of the females had mammary cancer but over 20 per cent eventually transferred the agent as shown by the development of cancer in their progeny.

It seems probable that these new observations from mice should be applied to any interpretation of human data on the possibility of the transmission of a milk-borne agent. Not only could such an agent be obtained from the milk but also it could be transferred by the male to the female, who, in turn, would pass it to the offspring in the milk. If it was present in the blood, as in mice, a transfusion would be a means by which an agent might be conveyed. Consideration must be given also to the possibility that it might be obtained by kissing. Although there is little evidence for a nursing influence for breast cancer in humans, in view of our present knowledge regarding the various methods of transmission of the "milk agent" in mice, it is doubtful if such an agent could be demonstrated even if one did exist in humans.

REFERENCES

Andervont, H. B.: *J. Nat. Cancer Inst.*, 1:147, 1940.
Andervont, H. B.: *J. Nat. Cancer Inst.*, 5:383, 1945.
Andervont, H. B.: *J. Nat. Cancer Inst.*, 10:193, 1949.
Andervont, H. B., and W. R. Bryan: *J. Nat. Cancer Inst.*, 5:143, 1944.
Andervont, H. B., and T. B. Dunn: *J. Nat. Cancer Inst.*, 8:227, 1948.
Andervont, H. B., and T. B. Dunn: *J. Nat. Cancer Inst.*, 9:89, 1948.
Barnum, C. P., and R. A. Huseby: *Cancer Research*, 10:523, 1950.
Bittner, J. J.: *Science*, 84:162, 1936.
Bittner, J. J.: *Pub. Health Rep.*, 54:1113, 1939.
Bittner, J. J.: *Cancer Research*, 4:779, 1944.
Bittner, J. J.: *A. A. A. S., Research Conference on Cancer*, pp. 63, 1945.
Bittner, J. J.: *The Harvey Lectures, Series* 42:221, 1946–1947.
Bittner, J. J.: *Cancer Research*, 8:625, 1948.
Bittner, J. J., and R. A. Huseby: *Cancer Research*, 6:235, 1946.
Bittner, J. J., and R. A. Huseby: *A Symposium on Steroid Hormones*, E. S. Gordon, Ed., Univ. Wisc. Press, pp. 361, 1950.
Bittner, J. J., R. A. Huseby, M. B. Visscher, Z. B. Ball, and F. W. Smith: *Science*, 99:83, 1944.
Dmochowski, L.: *Brit. J. Cancer*, 3:525, 1949.
Foulds, L.: *Brit. J. Cancer*, 3:230, 1949.
Gardner, W. U.: *Cancer Research*, 1:632, 1941.
Heston, W. E., and H. B. Andervont: *J. Nat. Cancer Inst.*, 4:402, 1944.

Heston, W. E., M. K. Deringer, T. B. Dunn, and W. D. Levillian: *J. Nat. Cancer Inst.*, 10:1139, 1950.

Huseby, R. A., and J. J. Bittner: *Unio Inter. contra Cancrum, ACTA*, 6:198, 1948.

Jackson Memorial Laboratory, Staff of: *Science*, 78:465, 1933.

Kirschbaum, A., M. Frantz, and L. Williams: *Cancer Research*, 6:707, 1946.

National Cancer Institute, Staff of: *A. A. A. S., A Symposium on Mammary Tumors in Mice*, 1945.

Samuels, L. T., J. J. Bittner, and B. K. Samuels: *Cancer Research*, 7:722, 1947.

Shimkin, M. B., and H. B. Andervont: *A. A. A. S., Research Conference on Cancer*, pp. 97, 1945.

Smith, F. W.: *Science*, 101:279, 1945.

Smith, F. W.: *Cancer Research*, 8:641, 1948.

Smith, F. W., and J. J. Bittner: *Cancer Research*, 5:588, 1945.

Woolley, G., E. Fekete, and C. C. Little: *Proc. Nat. Acad. Sci.*, 25:277, 1939.

Woolley, G., E. Fekete, and C. C. Little: *Proc. Soc. Exp. Biol. and Med.*, 45:706, 1940.

Woolley, G., E. Fekete, and C. C. Little: *Endocrinology*, 28:341, 1941.

Woolley, G., and C. C. Little: *Cancer Research*, 5:193, 1945.

Woolley, G., and C. C. Little: *Cancer Research*, 5:211, 1945

EXPERIMENTAL STUDIES IN METAL CANCERIGENESIS*

I. Nickel Cancers in Rats

W. C. Hueper

For more than a century arsenicals were the only metallic chemicals that exhibited upon occupational, environmental or medicinal exposure, causal relations to cancers in man. Observations made during the last two decades in the field of occupational cancerigenesis have more or less definitely incriminated several additional metals or metallic compounds, such as chromates, nickel carbonyl, and asbestos. Experimental investigations moreover have shown that beryllium and selenium are cancerigenic for certain species. To this list of cancerigenic metals there may be added the various radioactive metals of natural or synthetic origin that have produced cancers in man and/or experimental animals.

In view of the growing scientific and practical importance of metal cancerigenesis and the inadequacy or the complete lack of experimental investigations of this rather new facet of the cancer problem, a series of experimental studies were initiated dealing with the following metals or their compounds: arsenic, chromium, chromite ore, nickel, uranium, beryllium, and asbestos. In the selection of the technical approaches the following principles and considerations were applied:

The primary aim of the experiments was the demonstration of cancer producing properties of elementary metals. No attempts therefore were made to imitate in the experimental conditions (type of metal compound, intensity and kind of exposure) those met in human experience. The animals for this reason received, wherever necessary, maximal tolerated or injectable doses of powdered metals at sites where their slow resorption seemed to be assured (marrow cavity of femur, pleural cavity, and nasal sinus).

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Since human and experimental experience have shown that cancers develop at sites where cancerigens act over long periods of time even when present in only minute quantities, it was thought that the relatively low solubility of the metals in water and fat, would favor through a depot action their gradual release in concentrations that would not cause severe local metallo-toxic necroses, but rather a specific metallo-biologic action (metal catalysis, enzyme metabolism, metallic protein complexes, etc.).

For each substance tested several species (dog, rabbit, guinea pig, rat) were used so as to overcome, as much as possible, any species specific lack of susceptibility to the potential cancerigens.

In the selection of the particular species employed, care was taken to exclude species or strains possessing a marked genetic tendency toward the "spontaneous" development of cancers in organs or tissues in which experimental cancerous responses to the metals tested might be expected. Through this arrangement it was thought that any results obtained might be unequivocal, despite a smaller number of test and control animals used than that required when animals belonging to "cancer strains" are employed.

The present communication reports the experimental procedure used and results obtained in rats to which pure metallic nickel powder suspended in lanolin was administered by three different parenteral routes.

Experimental Procedures

Female albino rats of the Osborne Mendel strain were used. There were 25 animals 6 months old in the two series that received injections of a suspension of pure metallic nickel powder in lanolin into the right femur or into the right pleural cavity, while there were 20 rats 4 months old in the series which were given injections of such a suspension into the right nasal sinuses.

Intrafemoral Administration: The suspension of nickel powder in lanolin used contained 12.5% of nickel by volume and 61% of nickel by weight. Approximately 0.05 cc. of this suspension containing about 50 mgms. of nickel was injected into

the narrow cavity of the right femur. The suspension was warmed upon a water bath until it could be withdrawn through a 19 gauge needle into a 1 cc. tuberculin syringe. The needle was then inserted through a hole drilled into the distal end of the femur with the aid of a #1 dental drill activated by an electric motor with a flexible driving shaft (hobby drill). A second hole was drilled into the proximal part of the femoral shaft. The injection was continued until the grey black soft buttery material exuded from the hole in the proximal end. There was always some nickel suspension in the perifemoral tissue. The wound was then closed with sutures. The operation was performed under ether anesthesia.

Intrapleural Administration: Through the supraclavicular fossa 0.05 cc. of the same nickel suspension were injected into the right pleural cavity. These administrations were made in monthly intervals for a total of 5 injections.

Intranasal Sinus Injections: A nickel powder suspension in lanolin containing 12.5% of nickel by volume and 43.8% by weight was used. Through a 19 gauge needle 0.1 cc. of the suspension was introduced into the right nasal sinuses. The needle was placed for this purpose on the floor of the nasal cavity and its tip slightly raised so as to avoid the puncture of the palate. The needle was then gently pushed forward until a moderate bony resistance was felt. Under slight pressure this bony membrane was perforated and the needle further advanced until a firm bony resistance was experienced. The needle was then withdrawn for a very short distance and the suspension was delivered into the sinus cavities. As a rule very little bleeding from the nostril was met during or after the injection. Three such injections were made to each rat in intervals of 2 months between the individual injections.

Experimental Observations

Intrafemoral Injection Series: The surgical incisions healed well in all animals except one, in which the femur fractured and a fistulous duct was formed. Two rats died during the first 6 months of the experiment without showing gross evidence of tumor formation or any other symptoms referable to a metallotoxic or biologic effect. After about 8 months a

slight swelling of the right thigh was noted in one rat. During the following 8 months similar developments appeared in 3 additional rats. These tumors were firmly attached to the deep tissues but did not seem to involve the skin. They were firm in some parts and soft in others and grew rather rapidly. They extended in most instances into the pubic region and into the abdominal cavity. One became fixed to the spine and pelvic bones. They varied in size from that of a golf ball to that of a tennis ball. In one rat, with the fistula, however, there was a navy bean sized firm node beneath the fistulous opening and attached to the bone. In a fifth rat the right thigh was normal but the abdomen became increasingly distended by a tumor mass. These animals died or were sacrificed during the period of 7 to 16 months after the start of the experiment. During the same time 12 additional animals died without showing signs of neoplastic responses. There are six rats still alive. It appears thus that among the 17 rats that survived the minimal latent period of 7 months and died within 16 months after the injection 5 developed neoplastic responses, 4 involving the site of injection (30 per cent tumor incidence).

The post-mortem examinations of the rats showed that the tumors located in the right femoral region were rather well encapsulated toward the cutaneous surface, but at the same time were firmly attached to the femur. The involvement of the bone extended in one rat well into the pelvic ring and the spine. The tumor tissue on section exhibited usually a large central necrotic area, while the marginal parts were of homogeneous, white bacon-like texture. Pea sized to bean sized nodes of similar appearance and consistency were found in the abdominal lymph nodes and in the lung. In the rat with the abdominal tumor mass numerous smaller nodes were found in the mesentery and the retroperitoneal tissue. The rat with the fistula and the tumor surrounding this duct exhibited a second pea sized medullary growth located on the snout and forming there a projecting mass that was slightly adherent to the bone underneath.

In the rats which died without any neoplastic reactions a blackish material could be demonstrated in and around the right femur. One of them revealed a bean sized cyst filled

with debris and located in the muscular tissue beneath the original incision. The main pathology in these animals consisted of purulent pleurisy and pneumonia or enteritis.

The histologic examination of the tumors and tissues of the rats gave the following findings:

The sections taken from parts of the three tumors topographically closely related to the right femur and firmly attached to it show masses of irregular spindle shaped cells containing round, oval, elongated, hyperchromatic nuclei and originating from the periosteal tissue which contains a layer of newly formed osteoid and cancellous bony tissue (figs. 1, 2 and 3). The tumor tissue is found in close proximity to black

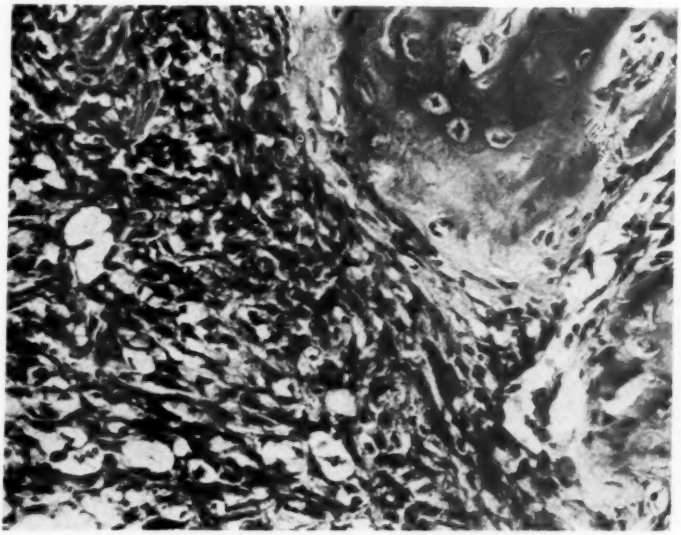

FIG. 1. R-435, Osteogenic spindle cell sarcoma of right femur.

nickel deposits. In distal portions of the primary tumors as well as in metatases the structural characteristics differ a great deal. In some parts the sarcomatous tissue is composed of irregularly arranged bundles of hyperchromatic spindle cells

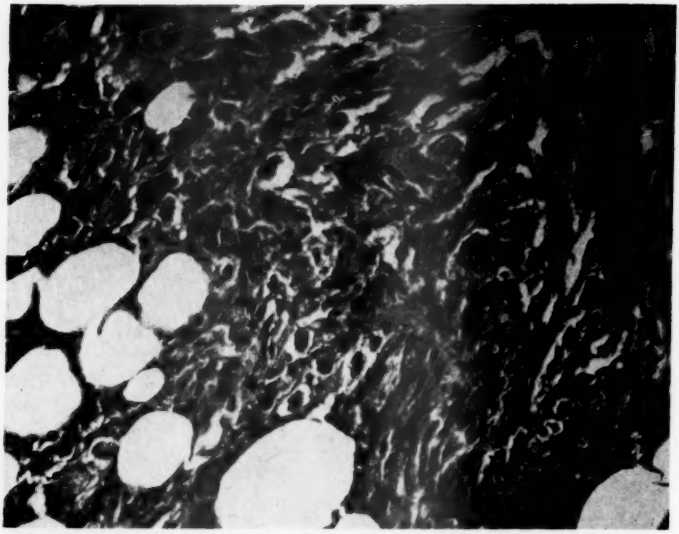

FIG. 2. R-432, Irregularly shaped round and spindle cells of a sarcomatous tumor originating from the femoral periost invading subcutaneous fat tissue.

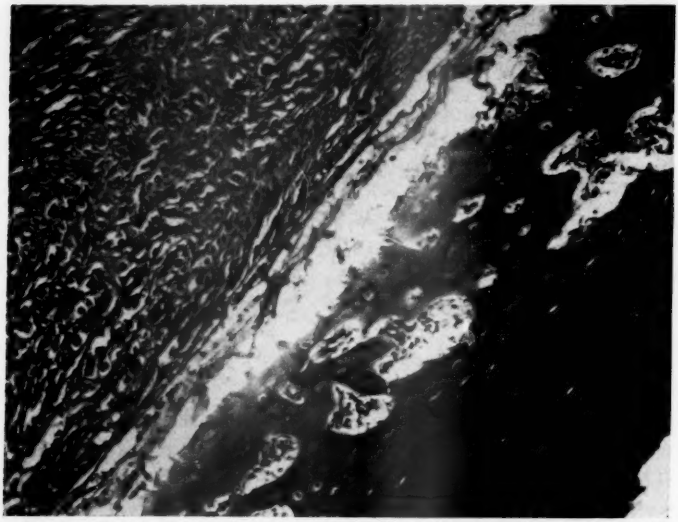

FIG. 3. R-422, Femoral cortex with periosteal spongy bone and spindle cell and round cell sarcomatous tissue.

having a moderate amount of fibrillar intercellular substance. Giant cells are scattered in this cellular matrix (fig. 4). The

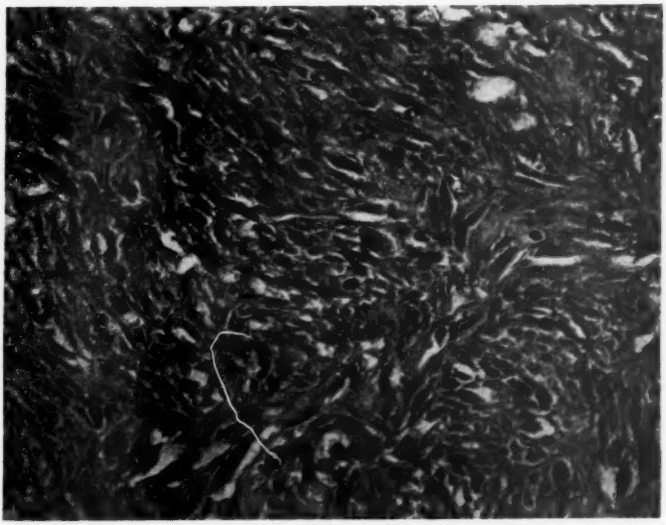

FIG. 4. R-422, Spindle cell and fibrosarcomatous areas with scattered giant cells.

nuclei are occasionally arranged in palissade formation. In other areas the tumor cells are irregularly round and show a dense scattering of huge cytoplasmic complexes containing small, hyperchromatic, nuclear bodies, resembling osteoclastic syncytial formations (fig. 5). In two cases parts of the primary and metastic tumors display large solid masses of indistinctly round cells arranged in small groups of ill defined, solid alveoli (fig. 6). Adjacent normal tissues, such as muscle tissue and fat tissue as well as mesentery, kidney, spleen, lung, pancreas and abdominal and mediastinal lymph nodes are diffusely invaded by tumor cells (fig. 7).

The tumor that seemed to originate in the abdominal cavity of one rat consists of masses of small round cells embedded in a scanty loose connective tissue. In some areas the tumor cells

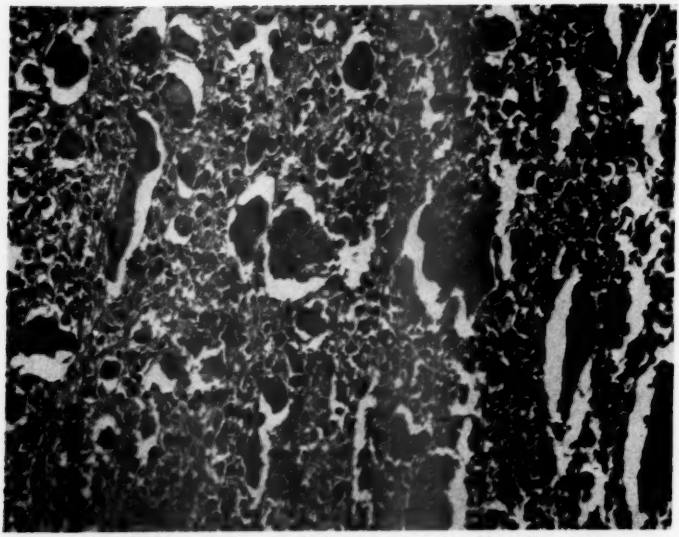

FIG. 5. R-422, Irregularly round shaped sarcoma cells with an admixture of large syncytial masses containing many small hyperchromatic nuclear bodies.

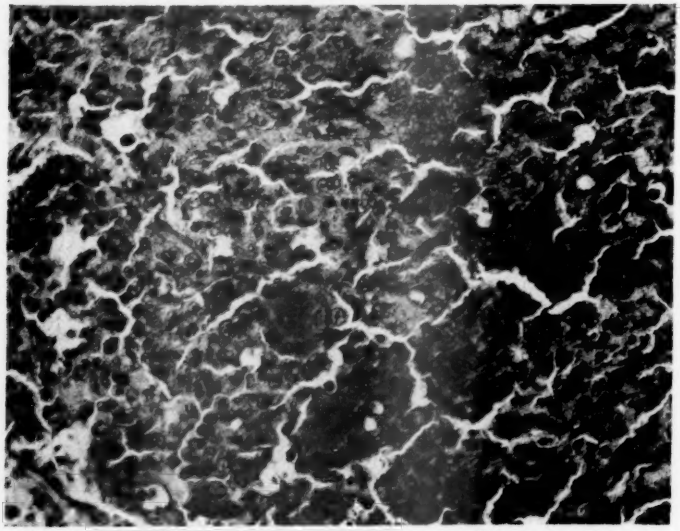

FIG. 6. R-435, Alveolar arrangement of large round sarcoma cells.

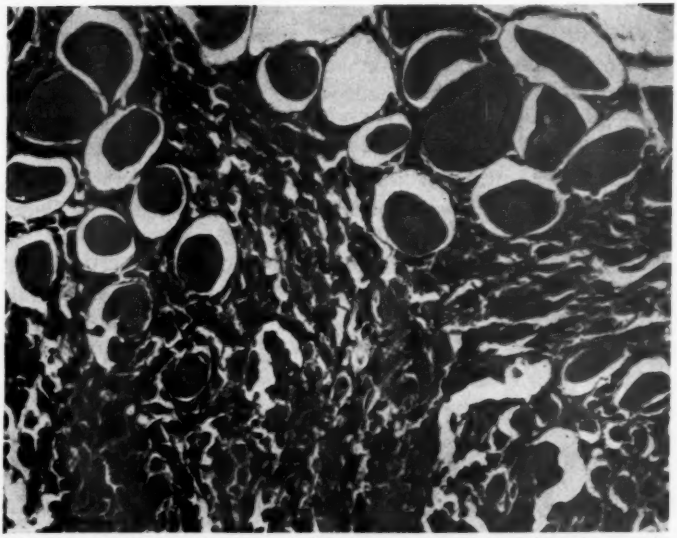

FIG. 7. R-422, Round and oval shaped sarcoma cells invading the muscle tissue of the thigh.

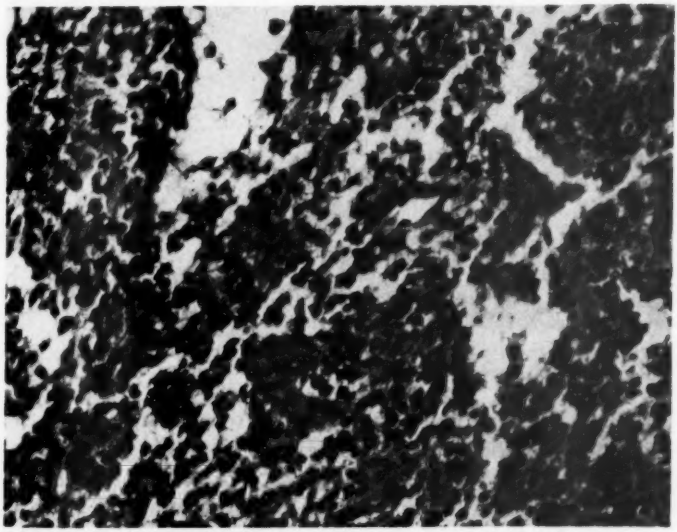

FIG. 8. R-434, Small round cell sarcoma apparently originating from abdominal lymph nodes.

display an abortive follicular arrangement. The femur cavity contains black amorphous material surrounded by a fibrous marrow and necrotic bone trabecles. The bone marrow located more distally from the nickel deposits consists of dense myeloid tissue, while the bone trabecles are hyperplastic.

Sections taken from the tissue surrounding the fistulous duct connecting the skin of the thigh with the femur, found in one rat, reveal irregular nests of small epidermal cells in a fibrous stroma. They extend from an atypical epithelial coating of the fistula. Periosteal proliferations of spongy bone at the base of the duct are invaded by these carcinomatous nests (fig. 9). The surface epidermis is normal. Deep seated

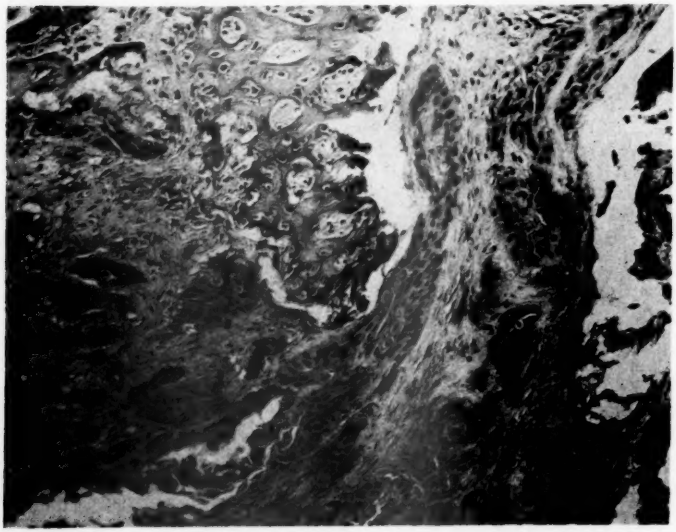

FIG. 9. R-429, Newly formed periosteal spongy bone surrounded by nests of epidermal carcinoma cells, extending from a fistulous duct.

carcinomatous cell nests are present in the subcutaneous tissue of sections of the small tumor node removed from the snout. The surface epithelium of the skin is normal.

The histological examination of the femur and muscle tissue of the right thigh of non-tumor bearing animals shows as a

rule some periosteal thickening and often a proliferation of osteoid and osseous tissue. The bone marrow is in general a dense myeloid tissue, that only in the immediate proximity of nickel particles exhibits necrotizing, atrophic and fibrosing changes. In a few instances osteoid tissue is present within the muscle tissue. This is usualy without any pathologic changes. The examination of all other organs (lung, heart, aorta, liver, spleen, pancreas, adrenal, kidney, sternum, stomach, ovary, salivary gland, bladder) shows occasionally abnormal alterations especially acute inflammatory reactions of the lungs and pleura as well as acute circulatory responses, all of which, however, do not bear any direct relation to the administration of nickel into the femur.

The administration of metallic nickel powder into the femur cavity of rats thus resulted within 7 to 16 months afterwards in the development of three osteogenic spindle cell sarcomas, one squamous cell carcinoma of a fistulous duct and, possibly, one round cell sarcoma originating in the abdominal cavity.

Intrapleural Injection Series: The injections were in general well tolerated. In two rats, however, sudden death occurred when the injection was given accidentally into the lungs. Following the fifth month there developed in the region of the right chest of three rats rapidly growing tumors which either extended downward into the sternum and abdominal wall or upward and backward involving then the spine. The tumors were rather firm and well fixed to the thoracical wall. The tumors varied in size from that of a large walnut to that of an oversized golf ball.

Of the 18 animals that died during the 16 months of the experiment post-morten examinations were performed on 12 rats. Six rats were destroyed by cannibalism. The autopsies performed on the three tumor rats show that the tumors are firmly attached to the bony structures of the thoracic wall (ribs, sternum, spine). The tumors are well encapsulated toward the skin. The cut surface reveals a homogeneous bacon-like texture. Black areas representing injected nickel are present within the tumor tissue of one case. Bloody serous fluid is found in the pleural cavity. In one rat white-yellow nodules are attached to the pleural surface of the lungs. The

cervical and axillary lymph nodes are enlarger, white, firm, and homogeneous in texture. The other organs are essentially normal.

The histological examination of the tumors and organs shows that the tumors are mainly composed of irregularly round and spindle shaped cells which are not infrequently of large size, have hyperchromatic round, oval or elongated nuclei and sometimes are intermingled with huge syncytial masses containing numerous small nuclei or larger chromatic masses (figs. 10, 11, and 12). There is evidence that at least in one case the tumor

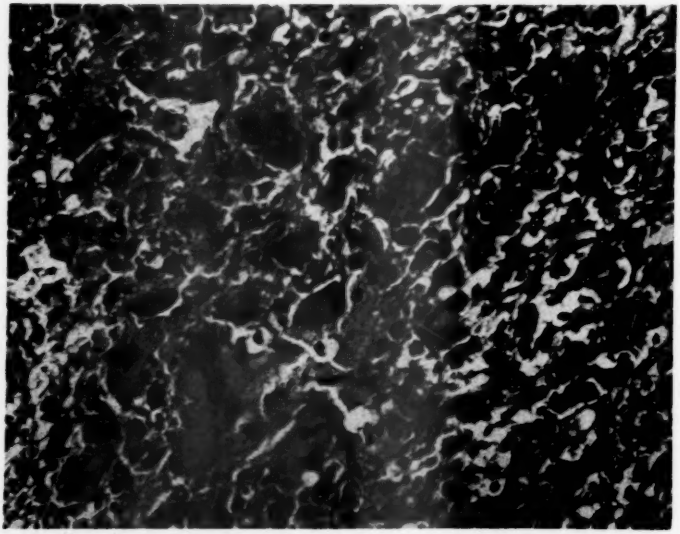

FIG. 10. R-445, Anaplastic sarcoma cells with a dense scattering of large giant cells having irregularly shaped, hyperchromatic, nuclei.

appears to originate from periosteal tissue of the rib, since there is a diffuse transition of cartilagenous and osseous tissue into round and spindle cell sarcomatous tissue (fig. 13). Nickel particles are demonstrable in close proximity to sarcomatous tissue. Round cell and spindle cell masses are found in lymph nodes, abdominal wall, lung and sternum. The histologic

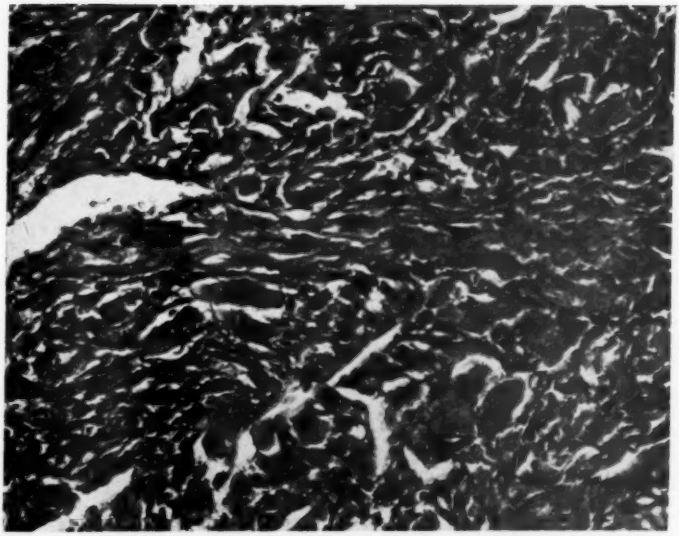

FIG. 11. R-446, Spindle cell sarcomatous tissue containing a moderate number of irregularly shaped large cells with a pink, homogeneous cytoplasm.

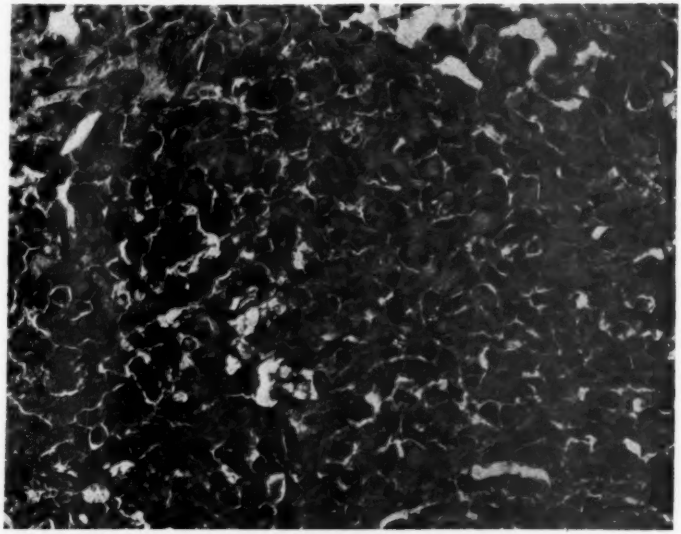

FIG. 12. R-449, Large round cell sarcoma.

FIG. 13. R-449, Periosteal portion of a large round cell sarcoma originating from a rib.

examination of the axillary tissue of a fourth rat which had not shown grossly any tumor formation reveals around black nickel deposits a proliferation of atypical, hyperchromatic, irregularly round and oval cells which represent apparently early sarcomatous foci elicited in the connective tissue by the presence of the nickel particles (fig. 14).

The findings of the histologic examination of the other organs of this series are without definite relation to the administration of nickel into the pleural cavity with the following exception. Black nickel deposits are present in the thoracical and pulmonary pleura where they are embedded in a highly capillarized granulation tissue forming brush-like excrescences. In two rats nickel particles surrounded by cellular granulomatous tissue are present in many pulmonary vessels (fig. 15). In two other rats, which died during the injection, nickel particles without perivascular inflammatory reactions fill many pulmonary capillaries.

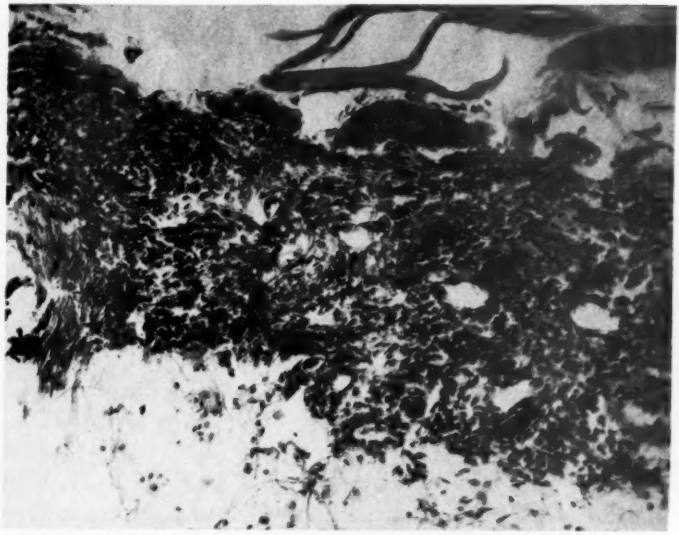

FIG. 14. R-455, Black nickel particles surrounded by hyperchromatic, irregularly round and oval cells suggesting an early stage of a sarcomatous transformation of mesenchymatous cells adjacent to nickel.

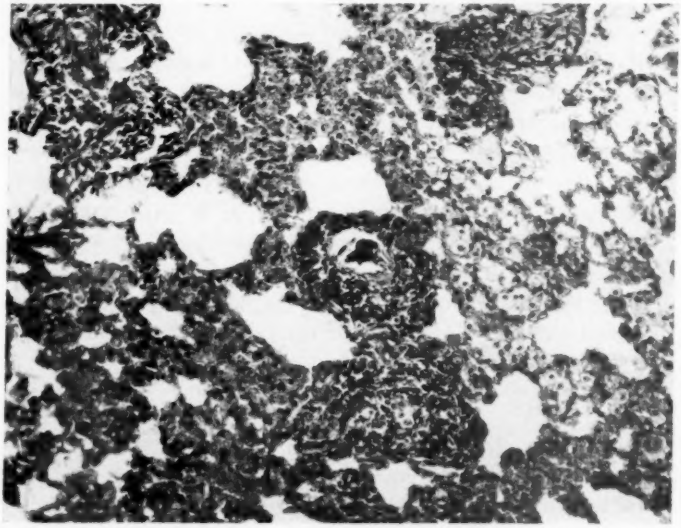

FIG. 15. R-429, Pulmonary tissue with intracapillary nickel particles surrounded by a dense mononuclear and lymphoid granuloma.

There were thus 4 sarcomas in the 10 rats that survived from 7 to 16 months the course of intrapleural injections of nickel powder into the pleural cavity. In all instances the tumors developed at the site of injection and were histologically similar to those observed in the thigh of rats injected intrafemorally with the identical material.

Intranasal Sinus Injection Series: The injections were in general well tolerated. Two rats died after the second injection. Several of the rats developed following the injections marked swelling of the snout, which, however, subsided after several weeks. In one rat this development led to the formation of a cystic pea sized mass on the dorsum of the nose. Of the 6 rats that have died during the first 8 months of the experiment 3 died 7 and 8 months, respectively, after the start of the experiment. One of these rats had a distended abdomen, while the second had the swelling of the nose.

The autopsy of the last mentioned rat shows a navy bean sized black nodule that is firmly attached to the skin and which reveals upon sectioning a necrotic cystic mass. The other organs including the nasal sinuses are grossly normal. In the second rat that died 7 months after the first injection the lymph nodes of the mediastinum are enlarged and hemorrhagic, the intestines are fused into a large mass including the mesentery and the greatly enlarged abdominal lymph nodes. These are dark red. The spleen is of normal size. The autopsy findings in the other rats show often purulent pleurisy and pneumonia. The nasal sinuses appear to be grossly normal. There are occasionally some small hemorrhagic spots in the brain.

Upon the histologic examination of the organs of this series the nasal sinuses exhibit often chronic inflammatory reactions. The cystic mass present on the nose of one rat consists of necrotic debris surrounded by an inflammatory wall. The enlarged mediastinal and abdominal lymph nodes found in a second rat are composed of solid masses of large round cells having a rather ample amount of cytoplasm (fig. 16). There is no neoplastic involvement of any other organ.

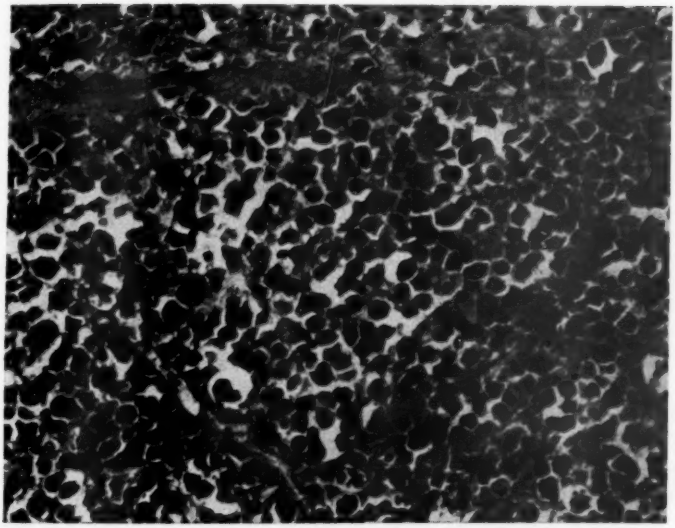

FIG. 16. R-641, Large round cell sarcoma originating from mediastinal or abdominal lymph nodes.

The intranasal sinus injection of nickel powder was followed in one out of 2 rats that died 7 to 8 months after the first injection by the development of a round cell sarcoma involving thoracical and abdominal lymph nodes. The neoplastic responses observed so far among the 70 rats which received by various routes injections of nickel powder are summarized in table 1.

TABLE I

Neoplastic Responses in Rats after Nickel Injections

Route	No. Rats	Deaths 0–6 months	Deaths 7–16 months	Tumor at Site of Inj.	Tumors remote from site
Femur	25	2	17	4	1
Pleura	25	8	10	4	0
Nasal Sinus	20	6	3	0	1

It appears from this listing that there were 10 malignant tumors among the 30 rats that died between 7 to 16 months

after the first parenteral administration of nickel powder suspended in lanolin. In 8 of these cancers the site of the cancer was closely related to the nickel depot produced. In several instances histologic examination revealed the proximity between nickel deposits and malignant growths. Of special significance concerning causal relations between the nicked deposition and the subsequently appearing cancer is the fact that one of the neoplastic reactions is of carcinomatous nature.

Comments

The scientific and practical importance of these observations lies in the fact that reports from English copper nickel ore refineries made during the past twenty years have shown an excessive frequency of cancer of the lung, nasal cavity and nasal sinuses among workers engaged in operations in which exposure to nickel carbonyl exists (Cooper; Amor; Bridge; Stephens). According to a recent report of Merewether there occurred between 1923 and 1948, 47 cancers of the nares and nasal sinuses and 82 cases of cancer of the lung among members of this occupational group. No similar observations so far have been recorded from Canada and Germany where the Mond Process of nickel ore refining is employed (Goldblatt and Wagstaff). While in previous years some investigators favored the view that exposure to arsenic containing sulfuric acid fumes generated during the refining process was responsible for these respiratory cancers, this view has been abandoned as untenable by practically all investigators in more recent years. Circumstantial evidence incriminates exposure to nickel carbonyl vapors, since inhaled nickel carbonyl is rapidly decomposed in the lungs into carbon monoxide and very finely dispersed metallic nickel which is precipitated on the respiratory surfaces, as well as nickel dust.

Experimental proof for this concept has been lacking up till now. Campbell exposed mice to the inhalation of nickel dust 6 hours a day, 5 days a week for one year and found increase of lung tumors of doubtful statistical significance. The experimental observations reported here appear to be of much more substantial nature as to the existence of a causal relation between an exposure to finely dispersed metallic nickel and the

development of cancer in the tissues where this metallic matter is deposited.

Although the total number of cancers observed among the rats treated with nickel powder is relatively small, the absence of similar tumors among 70 rats injected by the various routes noted with lanolin and among another series of 70 rats identically treated with injections of asbestos powder suspended in lanolin as well as among some 150 rats injected intramuscularly into the thigh with suspensions of various mineral oils and other organic chemicals incorporated into lanolin supports the view that the cancers observed in rats at the sites of injection of metallic nickel powder represent specific manifestations of experimental metal cancers. Some doubt in this respect, however, may be entertained in regard to the two round cell sarcomas that apparently originated from mediastinal or abdominal lymph nodes.

Conclusions

Three series of rats injected with a suspension of nickel powder in lanolin into the femur, pleural cavity and nasal sinuses exhibited cancerous reactions in 30 per cent of the animals that died from 7 to 16 months after the start of the experiment.

Of the total number of cancers observed 6 were apparently osteogenic sarcomas, 1 originated from connective tissue at the site of injection, 1 from the epithelial lining of an osteomyelitic fistula, while 2 seemed to have developed from abdominal lymph nodes.

The evidence obtained strongly suggests that finely dispersed metallic nickel may elicit in tissues of contact cancerous reactions.

The minimal latent period of the tumors observed is about 6 months.

BIBLIOGRAPHY

Cooper, E. H.: An important factor in the causation of industrial cancer. *Med. Press*, 187:397, 1933.

Amor, A. J.: Growths of the respiratory tract (preliminary notice). Ber. ü. d. 8. *Internat. Kongr. f. Unfallmed. u. Berufskrankh.*, vol. 2, p. 941,

1932. Nickel Carbonyl, in International Labour Office, Occupation and Health, Suppl., Geneva, 1938.

Bridge, J. C.: Ann. Rep. Chief Insp. Factories and Workshops of the Year 1936, H. M. Stationary Office, London, 1936. Ann. Rep. Chief Insp. Factories and Workshops for the year 1939. H. M. Stationary Office, London, 1941.

Stephens, G. A.: An Important Factor in the Causation of Industrial Cancer. *Med. Press*, 187:194 and 216, 1933.

Merewether, E. R.: Ann. Rep. Chief Insp. Factories and Workshops of the Year 1948. H. M. Stationary Office, London, 1949.

Goldblatt, M. W., and V. A. J. Wagstaff: Aspects of Industrial Medicine and Hygiene in German Chemical Factories. *B.I.O. Final Report* No. 1501, Item No. 24, pp. 11, 36.

Campbell, J. A.: Effects of Precipitated Silica and of Iron Oxide on the Incidence of Primary Lung Tumors in Mice. *Brit. Med. J.*, 2:275, 1940. Lung Tumours in Mice and Man. *Brit. Med. J.*, 1:179, 1943.

NOTE: When the experiment was terminated 24 months after its start there were 6 rats with cancers at the site of injection, and 2 rats with cancers in the abdominal cavity in the intrafemoral series, while 8 cancers of the site of injection had been observed in the intrapleural series. Two intra-abdominal cancers were found in the series given injection into the nasal sinuses. There was thus a total of 14 cancers at the site of injection; 42 per cent of rats surviving more than 6 months.

In new experiments with nickel obtained by the courtesy of the International Nickel Co., Inc., injected into femurs of rats in a gelatine base, sarcomas were formed at the site of injection within 4 months.

PROBLEMS IN THE ADMINISTRATION OF GRANT FUNDS FOR CANCER RESEARCH*

R. G. Meader, Ph.D., Chief, Grants and Fellowships Branch, National Cancer Institute, Federal Security Agency

It is a privilege to bring greetings from the Director of the National Cancer Institute and the Surgeon General of the United States Public Health Service to this Fifth Symposium of the M. D. Anderson Hospital for Cancer Research.

Since the major part of your program is concerned with advances in the knowledge of cancer control and cancer research, it may be appropriate for me to discuss with you some of the problems of facilitating work in these fields.

If there were time, I should like to acquaint you more fully with the organizational pattern and the functions of the National Institutes of Health and particularly of the National Cancer Institute, since it is through these units of the United States Public Health Service that your tax dollar is administered for cancer research and cancer control. I am constantly amazed by the fact that a large segment of the United States population, including physicians and scientists, is unaware of the national effort through their Government to control and prevent cancer and other diseases and conditions. They are also unaware of the availability of financial assistance to aid local efforts. I shall refrain from telling you about all this in the interest of time, but if anyone wishes to learn about these things, we at the National Cancer Institute shall be glad to supply the information.

Lack of adequate funds has long been the complaint and the excuse of the investigator. There is really little excuse today for failing to undertake first-class programs of research and control of cancer—at least on a small scale, for lack of money. There may not be available all the financial support one might desire to carry on larger-scale programs but, in

*Delivered at the 5th Annual Symposium of The University of Texas M. D. Anderson Hospital for Cancer Research, Houston, Texas, on April 20, 1951.

general, from one source or another funds can be obtained for promising investigations soundly conceived and planned by capable workers. It is in the allocation of these funds that some of the problems of which I shall speak in a moment arise.

Various estimates have been made of the funds available in the United States for cancer research. No one knows the exact amounts because of the number of local funds or endowments about which information is obtained with difficulty, if at all. The guesses as to the total cancer funds from all sources have ranged from 33 million dollars a year to 40 million or more. For cancer research alone as variously defined, estimates range from 12 to 15 million dollars annually, exclusive of special gifts or appropriations for construction of new laboratories and hospitals.

The total appropriation for the current year (1950–51) for all of the activities of the National Cancer Institute is a little less than 20 million dollars, including 5 millions in cash of funds previously authorized by Congress for construction of research facilities. No funds for construction beyond those already authorized are now available. Nearly 7 million dollars go to support cancer research in the laboratories and hospitals of the Nation's universities and research institutions, including those of the National Cancer Institute. Nearly 3 million dollars are used for education and training of present and future doctors and scientists (1) through the cancer teaching program (about 2 million dollars) in professional schools; (2) through the traineeship program ($359,000) for increasing the number of cancer specialists for the care of patients; and (3) through the research fellowship program (half million) for training the new generation of investigators. More than 4 million dollars are allocated for cancer control efforts (1) through the Health Departments of States (more than 3 million), and (2) by special control project grants (nearly 1 million). The balance is used for technical assistance to State and local health units, for demonstrations, for epidemiologic and statistical studies, and for those related to environmental or industrial exposure to actual or potential corcinogenic agents.

In sketching these activities and finances of the National Cancer Institute, I must emphasize that this is only one part of the total cancer effort. You are all very much aware of the American Cancer Society which enlists your voluntary dollars as the Congress does your tax dollars. You have heard this morning of a part of the cancer work being aided by the Atomic Energy Commission. In addition, private foundations such as the Damon Runyon Memorial Fund, the Jane Coffin Childs Memorial Fund, the Anna Fuller Fund, and many local organizations and individuals are important in providing financial support for cancer research and control, both within and without the United States. All of these agencies, including the National Cancer Institute, co-operate in many of their ventures and exchange information on others; to prevent undesirable and unnecessary duplication of effort, and so that the money available will be wisely and economically used.

One must be careful not to talk so much on how and what *organizations* do about cancer without mentioning the chief reason for their existence. Money, buildings, equipment, and all the rest are sterile without intelligent and trained brains to use them. Our greatest investment should be in the scientist. He should be as free as possible from economic want both in his personal life and in his scientific effort. Administrators should exist only to serve those who do the productive work. Suggestions and advice from a sympathetic and wise administrator may be helpful, but there should be constant awareness of the dangers of "directed" research.

What are some of the problems that must be met to give the best environment to the scientist?

In a democracy we want to be sure that both tax money and our voluntary gifts are used with all effectiveness and economy. We also want to see them distributed fairly and wisely. On this no one is more insistent than the citizen scientist. So we call upon committees of scientists and laymen to advise and guide the policies governing the distribution of moneys. For your tax money, designated for cancer, this is done by the National Advisory Cancer Council. The Surgeon General and his staff work closely with this Council, with other

Advisory Councils, and with research scientists and their institutions to develop the optimum conditions for research in the various fields aided by grants from the Public Health Service.

The problems arising in the course of this program are similar to those met by other agencies seeking the same goals. The solutions proposed have been many. Not always is it possible to apply a single solution both to the needs of the scientist and the financial or administrative needs of the institution.

When an agency or foundation is charged with administering a limited sum of money annually for specific areas of national scientific problems, the question is posed as to the best method to that end.

One method is to use the funds to establish institutes, like the Rockefeller Institute or the National Institutes of Health, which exert a national and international influence through the products of their research staffs and through the training of new investigators.

Another method is to distribute all or part of the funds directly or indirectly to institutions for the purpose intended.

If allocated indirectly, this could be done through State or regional organizations already existing or newly created, delegating responsibility to local groups and posing the question of criteria for distribution of the total among the local organizations to administer, and of the criteria to be used by them in the further allocation of funds.

If allocated directly or indirectly, should they go to all institutions equally or in some proportion to aid their staffs to accomplish more than the institutions' own funds will permit?

Should such sums be without specification except as to use within broad purposes, leaving to the individual institution the final decision as to how to use the money? Or should these funds be allocated by a central national representative group to particular institutions to aid the work of particular investigators on particular projects?

If funds are allocated to institutions for local decision as to specific use, should there be discrimination as to the eligibility of the institutions? Should all institutions of higher learning and research be eligible? Should the institutions where research

is now being actively conducted be most favored? Or should the institutions be favored that would like to foster and develop a research program but lack the aid with which to begin? It is often argued that one of the greatest sources of research potential lies in the institutions where young minds are being taught, but where little or no active research is underway.

If eligibility can be settled, what of the amount? Should all institutions share alike, or should there be discrimination based on factors such as size, research potential, research accomplishments, etc.? Could these be represented by variables and constants in a mathematical formula for distribution of funds to institutions in an equitable, sound and economical manner?

Could one use as quantities in such a formula the numbers of staff scientists; the number of projects in operation or planned for a certain period; the number of papers published; the number of reports at scientific meetings; the number of trips to meetings or to laboratories; the ratio of students to faculty (as indicating the time left for research by the teacher or the number of students being introduced to science); the number of specialized students being trained for the particular field to be financed; the need for funds, i.e., the ratio of local funds in relation to the research potential (however that may be determined)? Perhaps a value could be assigned to the geographical location to insure that each region, State, city, or even institution would receive a proportion of the funds regardless of other considerations, or in proportion to the population or to the taxes paid in that political subdivision.

From a set of variables and constants a mathematical wizard might derive a formula that could be used by clerks, or by an electronic "brain" to grind out the answer, and perhaps even write checks and mail them. This might provide considerable saving in money for administration of the program. The complicated and cumbersome methods necessary for subjective evaluations could be eliminated. And the secientists who now spend weeks on these tasks could return to the productive work of research and of training investigators, except that

they might then be called upon to perform for their own institutions the evaluating functions for distribution of funds which they had previously performed for national committees.

Some of this may strike you as being introduced facetiously but I assure you that most of it has been seriously proposed to me at some time by scientists, administrators, legislators, or laymen as arguments for favoring the institution in which they were interested. You will note that few, if any of these factors call for judgment on the quality of the staff scientists of an institution or of the significance of their research. It is often assumed that this assessment would be done by the staff scientists themselves if they have a chance to assist in the distribution of the undesignated funds thus acquired by the institution. Or it may be the dean or the director who will determine how and on what basis the funds shall be allocated.

This quantitative method of allocation of funds has not yet found favor with anyone I know when considered in its full picture. Parts of it might conceivably be combined with other qualitative and subjective values to make institutional grants for research which would be free from many of the objections leveled at the project type of support.

The alternative method of allocating available funds to particular institutions for particular investigators working on particular projects is based largely on subjective evaluation of the significance of a particular problem, the adequacy of the proposed techniques, the ability of the investigator to carry out the work properly, and the relation of the funds requested to the amount actually believed by experienced investigators to be needed to accomplish the job.

At the present time, the United States Public Health Service uses nearly all of the above described methods of administering the funds for which the Congress has made it responsible. That method is selected which seems best for the job to be done. Thus, through the National Institutes of Health and some other parts of the Public Health Service, research is carried out in Government-owned and operated facilities by Government employees. Funds for building of hospitals under the Hill-Burton Act are allocated to States by formula for assignment by a State commission or committee. Similarly funds for control of

cancer and other diseases and conditions are assigned to State agencies by a formula to help finance the local effort. But when it comes to research grants to nongovernmental institutions, the project grant method is that commonly employed by the National Advisory Cancer Council and by other Advisory Councils of the United States Public Health Service in determining the allocations to be recommended to the Surgeon General. The Committee on Growth of the National Research Council proceeds similarly in advising the American Cancer Society on the expenditure of part of its research grant funds.

It is of interest, however, that the American Cancer Society sets aside part of its total research funds to be granted in lump sum to some institutions for a cancer research program on a broad basis. Advisers to the Surgeon General of the Public Health Service are now giving attention to the desirability of augmenting its project type of support with the "institutional" or "block" type of grant.

The Congress provided by law that the Surgeon General should make grant awards only if recommended by a National Advisory Council, and that at least half of the appointive members of the Council should be physicians or scientists technically qualified in the medical and scientific aspects of the area of knowledge in which the Council is responsible for advising the Surgeon General.

Because of the wide range of detailed knowledge required for careful scientific review and because of the volume of projects proposed, the Surgeon General has invited more than 200 of the outstanding scientists from all parts of the country to sit as special technical committees called Study Sections to assess the scientific promise of research projects and to make recommendations to the Advisory Councils. This double review by Study Section and Council gives great assurance of fair and competent evaluation.

Furthermore, the members of both Study Sections and Councils serve limited terms and are replaced regularly by new members. This has the advantage of bringing new points of view to bear on problems and projects but it has the disadvantage of some loss of continuity because, each year, old or

continuation projects are new to some members of Study Sections and Councils.

Applications for grants should therefore be prepared with the realization that they will be carefully scrutinized by your fellow physicians and scientists and a thoughful, conscientious recommendation made. Sufficient detail of future plans and of past accomplishments must be given to permit this kind of study, always keeping in mind the type and amount of information you would want if you were asked to give your personal funds to another scientist to work on a problem or with techniques with which you were familiar.

While the competence of the investigator and the scientific merit of a project are paramount in securing a recommendation for a grant, the Councils are alert to opportunities for encouraging research in areas and institutions where special attempts are being made to initiate or expand research where little has been in progress.

Some investigators and many administrators object to project type research grants requiring applications that specify objectives, methods, budget, and the usual details requested by the conscientious scientists who review them. These restrictions, they feel, fail to provide for the exploratory type of study that is sometimes called "wild-catting" or "playing your hunches" because sufficient justification of such studies on the usual project basis is difficult to establish.

It is further argued that even when projects can be sufficiently specified to win a grant, the implied obligation to pursue the problem as outlined may prevent the investigator from following other promising leads that develop. Scientific research is said to be at its best when the imaginative investigator is free to follow the search for truth wherever it may lead without obligation to a previously stated project plan and budget.

There seems to be no agreement, however, as to how this freedom can be achieved and at the same time satisfy the desire of scientists and other taxpayers and donors to national fund-raising organizations to be assured that the funds they provide are allocated where they will be most effective and in such a manner that they will be available to help unproven

investigators as well as those who are better known. The established investigator suggests that he be given what he considers to be sufficient money and a free rein. The institutional administrator suggests that he be the arbiter of what constitutes the research in his institution meriting preferred support. Unfortunately neither is free from the criticism of his associates who may not have the same unbounded confidence in his wisdom and ability.

After listing a few more of the questions that confront those who are charged with the allocation and administration of funds for research, I should like to indicate how the United States Public Health Service has attempted to meet them and the other problems just mentioned.

How much assurance of continued support is necessary to give stability to the research of an investigator or of a team of investigators?

How long should such an investigator or team be given support if funds are insufficient to grant all requests?

Unless funds for expansion are made available, how do new applicants obtain support if previous grantees continue their work with a need as great or greater than the original grant?

Should new applicants have to wait for their support until the death of an investigator or some other event brings a research program to an end, or should the new proposals be allowed to compete with those previously approved in order to determine which should receive support? How can success in such competition be measured and what is needed to measure it?

Should the productivity of an investigator or of a research group be assessed to insure that the money is wisely used? If so, how should it be done and what criteria employed?

Should public funds for grants-in-aid for research be used to pay the salaries of senior investigators in view of the uncertainties that may affect the continuation of such support? If so, is it necessary to safeguard such grants from being used to relieve an institution from responsibility for the salaries of its regular personnel?

Should there be any limitation on the amount and type of equipment allowed as a charge against a grant? Should travel

funds be provided? If so, for what purposes—to attend scientific meetings in this country or abroad, to visit other laboratories to learn new techniques or exchange ideas or collaborate on research? Should there be any limitations on the amount, purposes, and kind of travel?

Is the purpose of national grant-in-aid funds primarily the short-term approach of securing the greatest amount of the products of research in the shortest period of time? Or is it the long-term approach of developing research potential, especially in those geographical regions or in those institutions where there has been less research done?

Should research grant funds made available for a special problem, such as cancer, be allocated according to some strict definition of what is "cancer" or should they support research in problems of normal and abnormal growth broadly defined?

Should the research support be limited to so-called "fundamental" studies or to what some call "applied" studies, or both?

How much of the indirect costs of research should be charged against a grant as "overhead" and how much borne by the recipient institution? Are these grant funds to be thought of as "buying" research results or as helping an institution to do what its funds are inadequate to do in providing for the research interests of its staff scientists?

These questions and many variations of them have led the National Institutes of Health to issue a Statement of Policy regarding most of them. I cannot hope to cover in detail all of these matters but a copy of the Statement can be secured from the Chief of the Division of Research Grants, National Institutes of Health, Bethesda, Maryland. I shall try to interpret some of them for you.

The purpose of the Public Health Service research grants is to facilitate progress in research in the fields of health by helping scientists to do work they cannot do otherwise through aid from their own institution or other sources of support. The aim is to get research done to improve the Nation's health, recognizing that this will require emphasis on the immediately solvable problems as well as upon development of the investigators, the techniques, the fundamental knowledge, and

the facilities necessary for the continuing successful attack on more elusive problems. This implies cooperation in the utilization of all available ready resources and in the development of new resources.

The procedure, thus far, has been to make grants for project type research, although the definition of a project is so elastic that some "project grants" would fit within some definitions of "program grants" or "institutional grants." The scientists on the Advisory Councils and Study Sections have found it difficult to evaluate vague and general broad "institutional" or "block" grant applications in competition with more specific projects. While the desirability and feasibility of some type of institutional grant is being studied, constant attention is given to eliminating the objectionable features of project grants.

In applying for a grant, the investigator is invited to be as specific as possible in describing his general research program, the part that he expects to be able to complete in the first year, the methods and procedures to be used and the extent to which they will require the personal assistance, permanent equipment, supplies, and other items in his budget. He is asked to show from the record of his training and of work previously done that he is qualified to carry out the proposed study. He is also invited to indicate how long he will need assistance to continue or complete the study and to estimate the probable sums needed in each of the additional years.

All of this is carefully studied by his fellow scientists in comparison with other proposals and other similar research known to be in progress. If a grant is recommended, the information provided may be sufficient to lead the Advisory Council to recommend support in some specific amounts for a definite number of years. The award of such a grant with future commitment gives assurance that if the Congress appropriates sufficient money each year to cover all the recommended commitments, and if satisfactory progress is made, the investigator may safely count on having for his research the amount specified by the Council for each year, if it is needed. Sufficient funds to honor these commitments will be set aside out of future appropriations before any grants are made for new

projects or for continuation projects whose commitments have been fulfilled.

If he has underestimated his needs, or if the Council has questioned his estimate and reduced his grant, the investigator may submit a request for supplemental funds in the same way as for an initial grant. No funds are reserved for such supplemental grants, however, and they must compete for the available balance of unallocated funds with all other such applications that do not have commitment.

The Council may reduce the amount of a grant and its commitment below that estimated by the applicant, usually because the application has not offered sufficient evidence that either the amounts or the time requested will actually be required. If the investigator has secured, or plans to secure, from other sources partial support for the project under consideration, he is asked to state what it is and the sources, so that the Council can review his request in the light of the total support he thinks he will need.

Unless the investigator requests only 1 year of support, or the Council believes the project is one that can be completed in 1 year or that there is need for exploratory work to indicate the need for longer support, there is a general policy that a new grant should be made for not less than 2 years. It is recognized that this is the only means by which the investigator can be given time to do enough work to show justification for a longer term. Moreover, each investigator is given an opportunity several months before the expiration of his final year of committed support to submit a request for future years. By this means he can learn the decision in time to allay the feeling of insecurity of his grant-paid assistants or to obtain funds from other sources if the Public Health Service grant is not renewed.

While these provisions do not carry complete assurance of indefinite support, such as many investigators would like and feel they deserve, they do go far to mitigate many of the objectionable features of annual project grants. And they do satisfy the demand for periodic review of progress and plans for the future which all consider essential for the proper stewardship of public funds.

While the investigator is asked to give as specific information as possible about his research plans he is free to modify those plans completely and follow any new lead once the grant has been made. This is to provide that freedom of research which is so necessary if we are to reap the harvest of unexpected results that occasionally lead the alert student to new discoveries. It would be a perversion of the intent of the grant if the researcher were to feel obligated to pursue the original plan described and to ignore new paths that have come into view. Obviously, he should be able to show, when continuation of support is requested, that he has used good judgment in altering his course.

Considerable latitude is allowed in adjusting budgets to the needs of the research as work progresses. The investigator may freely shift funds *within* budget categories as he finds it advantageous, without permission from the Public Health Service. He may also freely shift funds in a similar manner from any category into the categories for personnel and consumable supplies, provided the total of all category transfers within each grant year does not exceed 25 percent of the total grant. If there is need for transfers greater than that, permission may be granted by the National Advisory Council upon presentation of satisfactory explanation. Transfer into the categories of permanent equipment, travel, and other expense may not be made unless permission for these has been obtained from the appropriate administrative officer of the National Institutes of Health. This permission is quickly and readily granted if sufficient information is offered in explanation of the need and the total transfers do not exceed the 25 percent limit previously mentioned.

There is no arbitrary limit to the number of years that support may be provided for a project, to the amount of funds for a project, or to the total number of projects or amount of money that may be awarded to any one investigator or institution. Each instance is examined on the basis of all the information made available and in relation to all other requests.

However, to insure that new projects and new investigators will have an opportunity to compete for available funds, future support is usually limited to not more than 3 years. In

exceptional cases this has been made for 5 years or longer. When support beyond a committed period is requested, the application then made must compete with all other similar applications, with new applications, and with requests for supplements to existing grants.

It is not always easy to judge the promise of an initial application, but it is somewhat easier to judge the propriety of continuing aid to a study after reports of progress have been made. The most important ingredient of a progress report is the evidence that the work is being carried out in a sound scientific manner so as to produce valid data directed toward answering significant questions. Immediate dramatic results are not necessary to demonstrate successful progress, as all scientist reviewers are aware from their own experience. Ordinarily, a progress report is expected whenever an application is made for another year of support, whether that support has been committed or not. An exception is made in the case of the application for the second year of an initial grant with commitment for 2 or more years, because it is recognized there will have been only a few months of operation in which there will be few if any results to report beyond progress in the setting up of the experimental program. The progress report in this case is to be submitted when a third year or more of support is requested and when there may be some accomplishments to report. Such reports are important to guide the action of the Council. They should be fairly complete and include data to support the conclusions drawn, but as concise as possible to save the reader's time.

There is much conflicting opinion about the propriety and desirability of paying the salary of a mature investigator from a research grant. Many of those concerned feel that it engenders or abets an unstable and undesirable system of institutional expansion without institutional responsibility. This is viewed as having a bad effect on the morale and the research of the investigator, as reflecting discredit on the institution which may not be able to provide for a man when a project grant is not renewed, and often with unwarranted repercussions on the fund granting agency that for one good reason or another has withdrawn further support. Such terminations

are inevitable since most agencies do not intend their gifts to become permanent endowment but rather to aid the institution with the hope that it will eventually assume the financial responsibility. In the present financial plight of institutions whose income from endowment does not permit the assumption of these responsibilities, this situation becomes acute and no easy solution is evident.

Another point of view, is that the organizations desirous of expanding research should assume responsibility for the research man. For such organizations this would present a difficult problem unless their resources expand concomitantly, because assumption of such permanent responsibilities would soon tie up a large proportion of their funds and reduce the opportunities for encouraging new investigators and for aiding the exploitation of new discoveries. Moreover, most organizations feel that they must remain flexible to meet new developments and to permit those who later take over direction of their future to guide them in the directions that then seem desirable.

Some feel that the problem is becoming so great that only governmental resources will prove sufficient to provide that sort of a future which will attract first class minds into research. They predict a loss in the recruitment of such minds if there are many more examples of men who give their lives to research without appropriate recompense either in salary or security and finally reach the end of their apparent usefulness to society only to be discarded and to live in want. Whatever the cause, there are responsibilities which no group seems willing to accept. The problem remains unsolved but, in the meantime, every effort should be made to encourage institutions to take such men onto their permanent staffs.

With regard to grant allowances for permanent equipment and travel, the Public Health Service policy is to permit these to the extent that they subserve the efficient prosecution of a project. Travel funds are intended to facilitate research and not primarily to pay the expenses of attendance at scientific meetings whether or not papers are read. Interpretation of what facilitates research is left with the investigator and the administrative officials of his institution. Travel funds, like

the rest of a grant, are to be used with the same care and control as applies to similar uses of the institution's own funds.

Definition of research as pertinent to the cancer problem is as difficult as for any other field. For this the Public Health Service depends largely upon its advisers. Since these change periodically, since few persons are able to be consistent in their personal definitions, and since many points of view are involved, it is understandable that there is no clear and consistent definition to offer by which to determine whether "cancer research" support is to be limited to studies of malignant, invasive, metastasizing new growths in man and animals or is to be extended to all problems in any way related to the growth and differentiation or lack of it in cells of animals and plants. In many cases, the breadth of definition used is governed by the availability of grant funds in relation to the demand. In general, the answer is that all types of meritorious research should, if possible, receive support. Strict definitions should be avoided because they rarely fit all cases and still have to be interpreted. The same attitude applies to the question of "fundamental" versus "applied" research. Decisions must be reached on the basis of all facts available at the time the question is raised.

The matter of financing the indirect costs of research is one on which vehement opinions are expressed and which the Public Health Service is still exploring. For an institution with a large staff doing research, much of which is financed from external funds, the indirect costs may be considerable and may impose a heavy and unwarranted drain on resources intended to finance educational and other important and necessary functions. Such institutions, faced with reduced income from investments and dwindling unrestricted gifts and with expenses increased by inflation, argue that they cannot longer meet the burden of indirect costs imposed by research which they are urged to sponsor unless some source of additional finances is provided. They, therefore, plead that the agencies encouraging research expansion accept a greater share of this burden. While granting the seriousness of this situation, another point of view argues that the granting agencies are not "buying" research in the sense of contracting with the

institution to perform certain functions it would not otherwise be doing. Proponents of this view say the grants for research, such as the Public Health Service makes, are intended only to help the institution to enable its highly trained staff to do research they wish to do, and which they could not do otherwise. They point out that a great many tangible and intangible benefits accrue to the institution through these grants. These include the acquisition of new equipment, and of adjunct staff members who may contribute to the training of undergraduate and graduate students, as well as the retention of desirable personnel who might otherwise move to another institution where better research opportunities would offer greater inducement. They also emphasize the indirect and direct benefits that often result from the prestige of a larger program than could otherwise be financed. It is not infrequently reported that the receipt of external project grants has led to private gifts of money or land because of the recognition of competence which such grants have helped to provide. Whatever the solution that is finally reached, it is evident that it is not yet immediately at hand. The problem must continue to be studied.

I can take time to mention only one more of the many problems that plague the leaders of universities. This is the reported observation that the offering of research support through project grants sometimes leads to an imbalance between the research and teaching functions of an educational institution. Staff members who are successful in securing research grants may accept so much responsibility of this type that they neglect their teaching responsibilities. One remedy suggested by administrators of these institutions has been that money to hire teachers should also be supplied with research grants. Although Public Health Service policy permits the granting of funds to employ a substitute to carry the teaching and other duties of the grantee investigator under certain conditions, it is quickly evident that this is an unsatisfactory method of meeting the general problem posed. Obviously, the universities, colleges, and medical schools are in bad financial plight but their condition is so serious that grasping at the straws in the research grants will do them little good, whereas it would

quickly deplete support for research. A more general program of aid for educational institutions from voluntary gifts or from tax funds or both would be required to meet the problem. In specific cases of imbalance between research and teaching functions, the responsibility would seem to rest not on the granting agency but squarely on the shoulders of the institutional administrator. It is his business to see that the research and teaching functions are kept in balance. It is within his power, and it is his responsibility to see that the staff members do not accept grants that entail interference with their other duties. A wise and capable administrator can accomplish this.

I must end this with a word of thanks for your patience in sharing with me these thoughts on the problems that face any administrator of research grant funds. But in a very real sense they are his problems only because you have helped to create them. So they are your problems as well, and their solution has and will continue to have an intimate and direct effect upon your own lives and achievements. It behooves you, therefore, to give serious thought to them and to offer your suggestions as to how they can be solved for the greatest good for all—those who benefit from your discoveries, those who make the discoveries, and those who provide the funds. In the case of the funds from taxes and national voluntary collections, each of you may well represent that trinity. And in the case of cancer, there will be few of you who have not or will not in the future meet it as a personal matter of family or friends.

FACTORS AFFECTING SUSCEPTIBILITY IN CANCER OCCURRENCE*

C. P. Oliver

A few years ago, a lecturer made the statement that individuals can be divided into three classes with respect to cancer occurrence. These groups are: persons with an inherent predisposition so strong that they develop cancer regardless of circumstances; persons in whom predisposition is less developed so that they require exciting factors before cancer develops; and persons who have no predisposition to the disease and who would never develop cancer even though exposed to exciting factors.

The study of factors causing and affecting cancer occurrence in man would be made easier if we could separate individuals into the three categories indicated. However, the groups overlap to a considerable extent. Hereditary tendencies can be inhibited or alleviated. Hereditary resistance can be broken down. This is known from work with experimental organisms. It is, of course, more difficult to find means to control hereditary tendencies in man, primarily because our knowledge about heredity in man is sketchy.

Agents which precipitate, accelerate, or inhibit cancer occurrence are elusive. They may act as a stimulus to cause the manifestation of malignancy or to bring about cancer at an earlier age in one person, but fail to have such an effect in another. What these agents are and how, or to what degree, they operate in man are being actively studied. We are carrying on one such project with aid from the American Cancer Society and through the co-operation of Dr. D. Jackson who has a project with the U. S. Public Health Services.

Activators in Mice

Mice strains have been developed which differ in their hereditary tendencies to have tumors at specific sites. These

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strains may also vary in their responses to agents which influence the manifestation of this tendency. One active agent of importance in mammary tumor was found by Bittner (1936) in some genetic strains of mice. This is a strong incitor or activator of tumor growth but mice strains differ in their response to the agent. In strain C3H which normally has the milk agent, practically all of the females develop tumors if they receive the agent through their mother's milk. Strain C57 lacks the substance and the females have a very low mammary cancer incidence when nursed by C57 females. However, if C57 females are nursed by C3H females so that they receive the milk agent, some of them develop tumors. They are nevertheless more resistant to the milk agent than are C3H females and only about 50 percent of these C57 females develop tumors.

Even C3H females have a reduced incidence of mammary tumors if they do not receive the milk agent. Heston and co-workers (1950) removed C3H mice by caesarean operation and developed a subline, C3Hb, which by all tests is free of the milk agent. Among the descendants of this subline only 38 percent of breeding females develop tumors in contrast to 97 percent of the regular C3H females. A new set of conditions has developed with the C3Hb subline. They have the hereditary tendency but lack the milk agent; studies of this subline may help in understanding breast cancer occurrence in man. Cancer in C3Hb may occur in daughters produced by non-cancerous or cancerous mothers. There is only a slightly higher chance of a female with a tumor-bearing mother to develop a tumor than there is for one from a tumor-free mother.

The absence of the milk agent also retards the age of onset of mammary tumors. Heston (1950) reported the average tumor age for 71 affected C3Hb females to be 20.3 months. In contrast the average tumor age for the regular C3H females is approximately eight months.

Activators and Inhibitors in Man

The conclusive evidence that milk of some mice strains carries a substance which has the ability to cause mammary

tumors has caused some investigators to suggest that a like substance possibly occurs in man. So far the data do not clearly establish that human beings carry anything similar to the milk agent found in mice. It is entirely possible that the pattern for breast cancer in man is more similar to that in the agent-free C3Hb subline. Possible activators and inhibitors of cancer manifestation in man then become important.

Dietary restrictions apparently can have an effect upon tumors in mice. The frequency of tumors is reduced and the age of onset is retarded by restricted caloric intake. The effect is associated with a lessened gain in body weight. A relationship between controlled diet and cancer development in man is more difficult to study. Within a population food habits differ to such an extent that collecting accurate data becomes almost a hopeless task. Individuals differ in their interpretations of questions about their diet. Yet the subject needs more detailed study.

Racial groups, national groups, and social isolated groups frequently have widely varying relative cancer mortality rates, and especially when sites are compared. Several investigators have reported that Negroes have a lower cancer rate than do whites. Cancer of the cervix seems to occur at an earlier age in Negroes than it does in whites. The proportion of deaths from cancer of the uterus is reported to be considerably higher than deaths from breast cancer in the Japanese and Spanish people. That is contrary to the findings among some other groups of people.

Kennaway (1948), in a review of the literature on racial incidences of cancer, reported differences in rates of cancer mortality among five religious groups in the Netherlands. The data from this study are shown graphically in Figure 1. The four graphs represent the relative death rates for each religious group from cancer of the stomach, uterus, breast and all sites. "A" refers to the Dutch Reformed group; "B," to the Catholics; "C" to the Christian Reformed; "D" to the Lutherans; and "E" to the Jewish group. In each group the mortality rate from stomach cancer is the highest of the three sites studied. The groups tend to differ, however, in their comparative rates for the various sites. Groups A and D have the highest death

rate from gastric cancer and C has the lowest. Group A also has the highest rate for cancer of the uterus with B and D having close to that same rate; C and E have comparatively low rates. For breast cancer, D has the highest relative rate, with E being a close second, and C again having a comparatively low rate. Group "C" has the lowest rate for each site as well as for all sites.

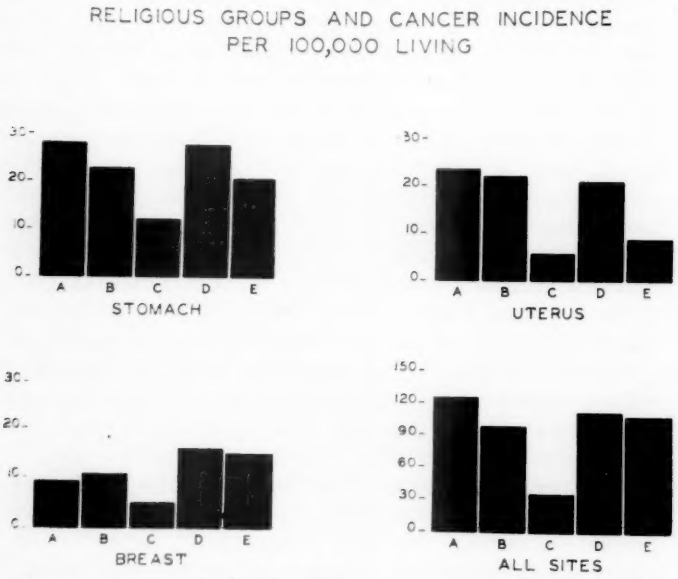

FIG. 1. A, B, C, D, E, identify religious groups, referred to in text. Modified from Kennaway.

Certain factors which may affect cancer occurrence and mortality rates should be investigated if a study of this type is to be of the most value. Marital status and child bearing seem to be important factors in the development of cancers of the uterus and breast. Social-economic status cannot be ignored. Differences in ages among the groups may be a factor. Death rates may vary among groups because of differences in medical care. Groups which are closely bound together in marriage by religion or custom may by inbreeding

become social isolates, each with its characteristic gene frequencies. If heredity is a factor, inbreeding isolates will then express their individual genetic differences.

National groups may differ in their familial tendencies for cancer occurrence and for ages at onset. An interesting set of conditions of this type can be found by comparing the data reported by Jacobsen (1946) with those by Penrose *et al.* (1948). The breast cancer women in Denmark who were used as probands by Jacobsen had a lower average age at onset if they were members of a familial case than did those who had no relative with breast cancer. Jacobsen also found that the proportions of breast cancer among the mothers and sisters of the probands and of cancer of all sites among mothers, sisters, fathers and brothers were higher than among the control groups. Busk (1948) computed the proportion of the relatives expected to have cancer if they faced the risk of the general population and supported Jacobsen's conclusions about the close relatives. Among the English probands used by Penrose, the average ages of onset for familial cases did not differ significantly from the average for the sporadic breast cancer cases. Penrose also found that the mothers and sisters of breast cancer probands had a higher breast cancer rate than was to be expected if they faced the same risk as the general population. This was not true, however, for cancer of other sites in the relatives of the probands.

Marital Status and Child-Bearing

The relative rates of mortality from cancer are strikingly different for the various marital classes (National Office of Vital Statistics, 1950). Among the middle age and older groups, the lowest cancer mortality is found for married men and women, and the highest occurs among divorced persons. Mortality rates for single persons are in general between those for married and divorced individuals. Death rates from breast cancer are higher, after age 34, for single and divorced women than for married and widowed women.

In many reports summarizing breast cancer cases, single women occur in greater proportion than they represent in the

general population. The percentages of single, married and widowed women among breast cancer cases reported by Harnett (1948) are included in Table 1. Single women, of all

TABLE I

Marital Status and Cancer of Breast and Uterus. Percentage of Persons in Each Status

Cancer Site	Breast		Cervix		Corpus	
Source	Harnett	Control	Harnett	Speert	Harnett	Speert
Marital Status						
Single	22.1	17.1	4.0	6.0	22.9	18.0
Married	58.0	63.1	75.6	75.0	52.5	65.0
Widowed or Divorced	19.8	19.8	20.4	19.0	24.6	17.0
Total %	99.9	100.0	100.0	100.0	100.0	100.0
Number of Cases	2129.	509.	856.	603.	288.	232.

ages, represent 22.1 percent of 2,129 cases. This is significantly higher than the proportion of single women in a control group referred to by Harnett. Penrose and co-workers (1948) had a similar high proportion of single women among 510 cases. This high frequency is not always found. Oliver (1950) reported that only 7 percent of 201 indigent breast cancer patients in Minnesota and 13 per cent of 117 non-indigent cases in Texas were single women. This second group has been increased to 220 cases with 8.6 percent being unmarried. Comparative control populations for these cases have not been completed. Widows in the report by Harnett occur with equal frequencies in the breast cancer and control groups.

Cases of cancer of the cervix and body of the uterus, included in Table 1, are taken from reports by Harnett (1949) and by Speert (1948). In contrast with breast cancer cases, cervical cases include a very small proportion of single women and a large proportion of married women. The proportion of widows does not differ significantly from that for breast cancer cases. Maliphant (1949) reported a series of cervical cases in which he separated the groups only according to single and married status. Only 1.3 percent of 1,114 women were single. In the community from which the cases came, 10.5 percent of women in the cancer risk age groups were unmarried.

The two reports, in Table 1, on cancer of the body of the uterus differ somewhat for the relative proportions of women in the three marital states. Speert found a distribution which is in agreement with the data shown for controls of the breast cancer study. Harnett's cases of corpus cancer show a smaller proportion of married women and an increased frequency of widows in comparison to the breast cases. It is not possible to explain the differences between the two reports.

Marital status is an important factor in cancer occurrence in these three sites. Marriage decreases the chance that cancer of the breast or of the body of the uterus will occur. It increases the hazard that cancer of the cervix will develop. There is a strong social factor in cervix cancer, nor can the social status be eliminated from any study of breast or corpus cancer.

Child bearing seems to have a beneficial effect with respect to cancer of the breast and of the body of the uterus. Its effect is just the opposite in cancer of the cervix. Data taken from several reports and modified to show the percentages of childless and fertile married women with cancer of the breast, cervix and corpus of the uterus are given in Table 2. The

TABLE II

Percentage of Childless and Fertile Married Women

Cancer Site	BREAST			CERVIX				CORPUS	
Source	Penrose	Harnett	Our Data	Maliphant	Control	Speert	Harnett	Speert	Harnett
Childless	22.3	16.1	21.4	5.1	14.5	17	10	30	21.8
Fertile	77.7	83.9	78.6	94.9	85.5	83	90	70	78.2
Total %	100.0	100.0	100.0	100.0	100.0	100	100	100	100.0
Number of Cases	408	1658	201	624	3182	567	727	191	202

last line includes the number of married women used from each report. Penrose and co-workers (1948) reported that 22.3 percent of their married cases were childless. The proportion of sterile women in our data is very similar to that found by Penrose. Harnett (1948) had a lower frequency of childless married women in his breast cancer group but the proportion, 16.1 percent, is higher than the proportion, 12.5 percent, he referred to as a control group.

Cancer of the body of the uterus in women reported by Harnett (1949) is distributed between the fertile and nonfertile groups in proportions approximating those observed by Penrose for breast cancer. Speert (1948) did report a higher frequency of childless married women in his group.

Cancer of the cervix reported by Harnett (1949) occurs in a greater proportion of women who have borne children than is true for breast cancer cases. Speert's report (1948) shows a higher frequency of childless women than is found in Harnett's data, but the proportion is still low when compared to breast cancer. Maliphant (1949) included in his report a comparison of the fertility of the women with cervical cancer and wives of unskilled laborers in the community who were in the same age groups as the cancer cases. The results, given in Table 2, show that the cervical cases have a higher proportion of fertile married women, the respective proportions being 94.9 and 85.5 percent. Maliphant concluded that if a woman reaches age 35, her risk of developing cancer of the cervix is doubled if she is married and increased ten times if she has children.

Marital status, social status, and childbearing are factors which must be considered when groups of individuals are compared with respect to cancer of the breast, cervix or body of the uterus. There is strong suspicion that the number of pregnancies or of children may also be important.

The average number of progeny for the various cancer types, as reported by several workers, is given in Table 3. Proper controls are lacking for each set of data. Speert reported an average of 1.34 births for 232 corpus cases. If only those

TABLE III

Average Number of Children by Cancer Patients

Source	Cancer Site	Number Cases	Average No. Children	Cases with Children	Average No. children per fertile woman
Speert	corpus	232	1.34	136	2.28
Speert	cervix	613	2.82	475	3.58
Maliphant	cervix	649	4.0		
Control		5016	2.4		
Penrose	breast	510	1.54	321	2.44
Our Data	breast	220	1.81	152	2.68

women who had at least one child are considered, the average is 2.28. According to the data he reported for cervix cancer cases, the affected women had a larger number of children than did women with corpus cancer. The average was 3.58 per fertile woman. Maliphant reported an average of 4.5 children for 1,182 women with cervical cancer. For purposes of comparing his data with that collected from a suitable control and recognizing that cervical cancer is more likely to occur among the poorer members of a community, Maliphant compared the average parity of the more recent cancer cases with that of the lower income groups. Cervical cancer cases had an average of 4 children as compared to 2.4 for the control.

Breast cancer cases have fewer children than do the cervical cancer cases but more progeny than the corpus cancer cases have. Penrose reported that the 510 women in his study had an average of 1.54 children. The average for 321 women having at least one child was 2.44. The average number of births for women in London of the same ages as the cancer patients was 1.63. Our breast cancer data include 220 non-indigent patients. They have had an average of 1.81 children, or for 152 fertile women the average is 2.68. Seventy-four percent of the 152 childbearing women had more than one child. Data on the fertility of non-cancerous sisters of the cancer patients are being collected for one control. Those women who have left the social class of their sister patients will be separated out.

Factors Affecting Age of Breast Cancer Onset

The frequency distribution for age of onset of breast cancer cases tends, for some reports, to take the form of a bimodal curve. Anderson *et al.* (1950) have summarized the frequency distributions for data collected by investigators working with different national and geographic groups of people. Where the distribution is shown in 5-year intervals of age at onset, one peak occurs in the 45–49 age group in 4 of the 5 sets of data; in the fifth, the 50–54 age group has the first peak. A second peak is observed in the 60–64 age group in 3 sets of data; five years earlier in the fourth; and somewhat irregular in position in the fifth set of data. If our 220 cases are added to

those summarized by Anderson, the bimodality is not affected. It has been suggested that the bimodal distribution may be an expression of the effect of conditions at menopause on breast cancer occurrence. A study of the rate of breast cancer incidence shows a rapid increase in the incidence up to the 45–49 age interval. This is followed by a slower increase until age 55 after which the rapid increase again occurs.

This information still leaves unknown those factors which cause some women to have cancer at an early age and others to live almost the complete expected span of life before the disease occurs. Harnett (1948) reported that marital status may lead to a later age of breast cancer onset. He found that the average onset age for 471 single women was 55.6 years. For 1,658 married women, the age was 57.4. The difference, 1.8 ± 0.66, is within the realm of being significant. Our own data include only 19 single women. The youngest single woman with breast cancer was in the age group of 30–34 years; the oldest, 75–79. Their average age was 49.2 years which is lower than the 52.9 years for the married women, but the difference can not be considered significant.

Lack of pregnancies in our data seems to have little if any effect upon age of breast cancer onset. Sixty-five childless single and married women were found to have an average onset age of 51.7 years. The difference between the average age for those having at least one pregnancy, 53.2 years, and the average age for the sterile women is less than twice the standard error.

One will find that the average age of onset for women with from one to three pregnancies is earlier than that for women with from 4 to 6 pregnancies. The age for women who have 7 or more pregnancies is even later, but our 220 cases included only 7 women with more than 6 pregnancies. A test for independence between age of breast cancer onset and number of pregnancies was made. For this preliminary test, a wide range of age intervals (15 years) and a grouping of number of pregnancies into two classes (1 to 3, 4 to 6) were used. The small number of pregnancies exceeding 6 were omitted. The results indicate that the ages of onset and number of pregnancies, as grouped, are probably independent ($X^2 = 4.924$; P close to 0.20). This also holds true for number of live births and ages

of breast cancer onset grouped in the same categories as were the pregnancies.

Conclusions

References have been made to some of the agents or factors which seem to be associated with variations in rate of mortality from cancer, and which possibly accelerate or inhibit cancer. Information about factors which influence cancer occurrence is of considerable importance to mankind and numerous investigations have been made of the problem. Yet very little is known about how or to what degree various agents can affect a tendency for cancer to develop.

Certain sets of conditions are recognized as having some association with high and low cancer mortality rates. Racial origin is important. Some races have distinct differences in rates of death from cancer. They frequently differ with respect to rates for specific sites. National groups and subpopulations, where the members are by custom isolated from other groups, show differences in rates of mortality from cancer of specific sites and in some cases from total cancer mortality.

More specific factors can be shown to have some association with cancer rates. Relative longevity can be a factor in racial, national, and subnational differences in cancer mortality. Differences in marital status of groups to be compared cannot be ignored. Marriage seems to be responsible for a decrease in rate of cancer of the breast, body of the uterus, and some other sites. Marriage increases the possibility of cancer of the cervix. Closely tied in with marital status is the social-economic status of the group. Cervical cancer apparently occurs with a definitely higher frequency in lower than in higher social groups. But women in the lower economic groups also have more children and possibly at an earlier age. Bearing of children increases the likelihood of death from cervical cancer and decreases the possibility from breast cancer.

Factors affecting the age of cancer onset are less easy to establish. The frequency distribution of cancer ages takes the form of a bimodal curve, according to some data. This fact is related to a retarded cancer incidence about the age of

menopause, but the range in cancer onset is from 20 to 80 years of age. Positive family histories of breast cancer seem in some groups to be associated with an earlier age of onset. Yet other people fail to fit that pattern, and the age of onset for breast cancer cases with similarly diseased relatives does not differ significantly from the age for cases with no known relative having breast cancer. Women with a large number of children tend to have a later age of breast cancer onset than women with few progeny. The relationship between total number of pregnancies or live births and age of breast cancer onset requires more detailed study.

REFERENCES

Anderson, E., S. C. Reed, R. A. Huseby, and C. P. Oliver: Possible relationship between menopause and age at onset of breast cancer. *Cancer*, 3:410–414, 1950.

Bittner, J. J.: Some possible effects of nursing on the mammary gland tumor incidence in mice. *Science*, 84:162, 1936.

Busk, T.: Some observations on heredity in breast cancer and leukemia. *Annals Eugen.*, 14:213–229, 1948.

Harnett, W. L.: A statistical report on 2,529 cases of cancer of the breast. *Brit. J. Cancer*, 2:212–239, 1948.

Harnett, W. L.: A statistical report on 955 cases of cancer of the cervix uteri and 321 cases of cancer of the corpus uteri. *Brit. J. Cancer*, 3: 430–473, 1949.

Heston, W. E., M. K. Deringer, T. B. Dunn, and W. D. Lavillain: Factors in the development of spontaneous mammary gland tumors in agent-free strain C3Hb mice. *J. Nat. Cancer Inst.*, 10:1139–1156, 1950.

Jacobsen, O.: *Heredity in Breast Cancer*. 306 p. 209 Ill. London. H. K. Lewis and Co., 1946.

Kennaway, E. L.: The racial and social incidence of cancer of the uterus. *Brit. J. Cancer*, 2:177–212, 1948.

Maliphant, R. G.: The incidence of cancer of the uterine cervix. *Brit. Med. J.*, #4613:978–982, June 4, 1949.

National Office of Vital Statistics: Cancer mortality by marital status: United States, 1940. *Special Reports*, 32:206–235, 1950.

Oliver, C. P.: The use of genetics in cancer research. *Texas Reports on Biology and Medicine*, 8:183–206, 1950.

Penrose, L. S., H. J. Mackenzie, and M. M. Karn: A genetical study of human mammary cancer. *Annals Eugen.*, 14:234–266, 1948. (Also 1948. *Brit. J. Cancer*, 2:168–176.)

Speert, H.: Corpus Cancer: clinical, pathological, and etiological aspects. *Cancer*, 1:584–603, 1948.

MOTION PICTURE STUDIES OF LIVING PAPILLOMA OF THE BREAST AND BREAST CANCER*

C. M. POMERAT

I presume to bring very fragmentary data before you because I believe we are in the process of developing promising techniques which may be of value to other investigators and because it is important in the course of these meetings to visualize some of the behavioral features of living normal and tumor cells from the human breast. Moreover, since it is well established that many varieties of normal and pathological cells can be maintained in large quantities in continuous culture (cf. Pomerat, 1951), this source of experimental material should not be overlooked. At a previous meeting of this Symposium, it was pointed out that free cells from pleural and peritoneal fluid provide an ideal study object for investigations on the action of chemicals, particularly sterols, involving long-time observations under highly controllable conditions (Pomerat, Nowinski and Rose, 1950).

The present study dealing with tissue cultures of the breast was originated by and is being conducted in collaboration with Dr. Dudley Jackson and Dr. David A. Todd of San Antonio. The early literature dealing with the cytology of nipple secretions together with a preliminary report on results obtained with the use of a smear technique utilizing significant criteria for their evaluation has been described by Jackson and Severance (1946). In a lecture presented at the meetings of the Chicago Tumor Institute, Jackson, Todd and Gorsuch (1951) reported on results obtained on more than 6,000 smears of breast secretions from 3,500 women. The value of this diagnostic procedure is enhanced by the opportunity it affords in exploring possible relationships of the silent papilloma to frank carcinomas.

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*From the Tissue Culture Laboratory, Medical Branch, The University of Texas, Galveston, Texas. Presented at the Fifth Annual Symposium on Fundamental Cancer Research at The University of Texas M. D. Anderson Hospital for Cancer Research, in Houston, Texas, on April 20, 1951.

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Materials and Methods

Cultures were made on #1 thinness cover glasses measuring 12 x 50 mm. Fragments of breast tissue cut approximately 1 mm. square as well as cells in fluid collected from the nipple were imbedded in a medium consisting of equal parts of heparinized cockerel plasma and extract derived from 7–8 day chick embryos. As soon as the clot had set, the slides were placed in Pyrex test tubes measuring 15 x 150 mm. and closed with sterile cotton stoppers. After standing for 1–2 hours, 2 ml. of nutrient fluid was added. This consisted of 50% human ascitic fluid, 45% Gey's balanced salt solution and 5% embryonic extract. Sodium Penicillin G was added to make a final concentration of 1,000 units per ml. Following the addition of the nutrient fluid, the tubes were tightly closed with #0 rubber stoppers and placed in an almost horizontal position in a drum designed to complete 8 revolutions per hour.

While it is difficult to obtain much information by direct examination of cultures on slides within such test tubes, it is possible to determine the moment and general character of migrating cells with the use of a 32 mm. objective and a 10 x ocular, using an ordinary compound microscope. Slides can be removed at regular intervals for still and motion picture photomicrography as well as for staining. A phase contrast setup (AO) utilizing 8 and 1.9 mm. (dark) objectives was used for the study of living preparations. Cinematographic records were made of some preparations with a time-lapse device (Rolab Company) at a rate of 8 frames per minute. Since these are projected at 16 frames per second, the activity seen in the films is accelerated by a factor of 120 times.

Plate 1

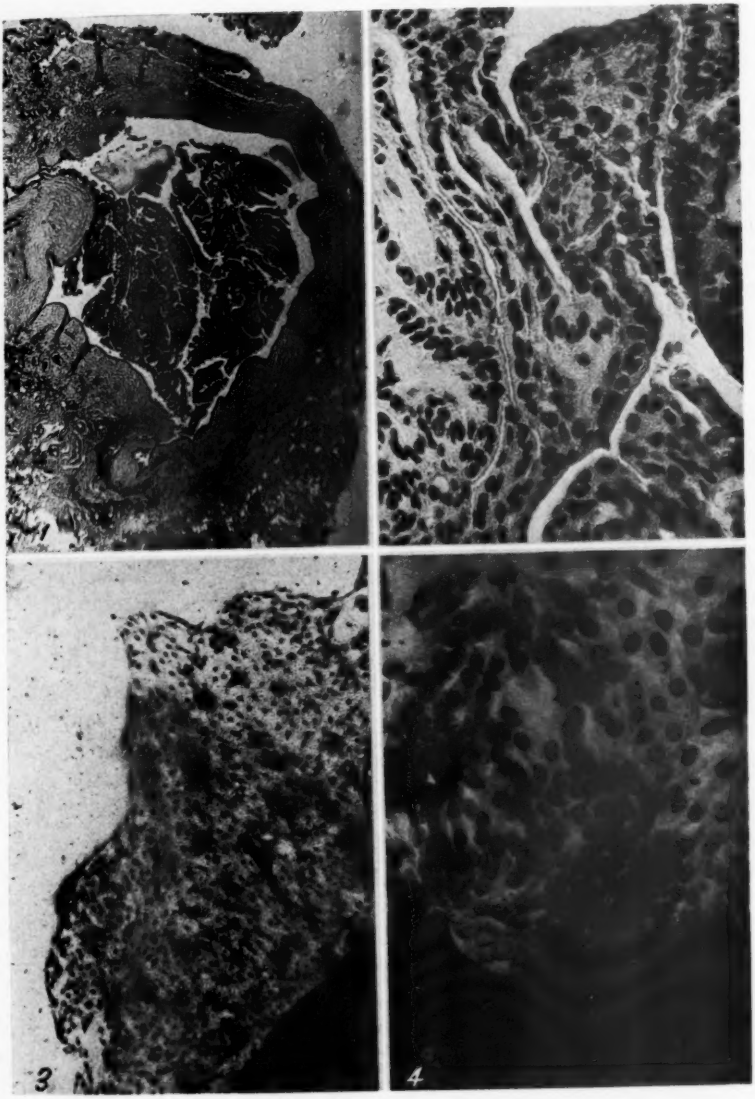
Figs. 1–4. Intraductal Papilloma of the Breast Patient. M.M. JSH Path. No. S28491. Age 36, white female.

Fig. 1. Section of nipple duct showing luminal papillomatous tissue *in situ*. 25x. Hematoxylin-eosin.

Fig. 2. Same as figure 1 but at 250x, showing characteristic arrangement of nuclei.

Fig. 3. Epithelial outgrowth from intraductal tissue shown in figures 1 and 2 after 12 days *in vitro*. 50x. Jacobson's stain.

Fig. 4. Same as figure 3 but at 250x. Note general similarity of nuclei.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Following fixation with absolute methyl alcohol (5 min.), slides were stained with Jacobson's method which consists of the May-Grunwald mixture followed by Giemsa.

Intraductal Papilloma

Figures 1–5 are presented to illustrate results obtained from the cultivation of an intraductal papilloma. The position of the papillomatous tissue within a duct near the nipple is shown in the histological preparation at low (fig. 1) and at high (fig. 2) magnification. Nuclei were generally found to be of regular size, shape and chromaticity. Outgrowth from explants of this tissue after 7 days of incubation is shown in figure 3. The arrangement of the cells adjacent to the explant is represented in figure 4. While there are irregularities in concentration of nuclei associated with the migration of the epithelial sheet, their appearance closely resembled that which was found in the section of the specimen.

The margin of cells at the edge of the epithelial sheet showed ruffles associated with cytoplasmic vacuoles (fig. 5). Time-lapse cinematographic records showed marked pinocytosis (cell drinking) in the wake of membrane activity of the ripple type extending over a relatively broad segment of the cell margin, resembling that reported for alveolar cells from the lung of the salamander studied with similar technique (Danes, 1949).

Nipple Secretion

Success in establishing cultures of exfoliated cells from prostatic secretion (Cone, Hooks, Pomerat and Rose, 1949) has

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PLATE 2

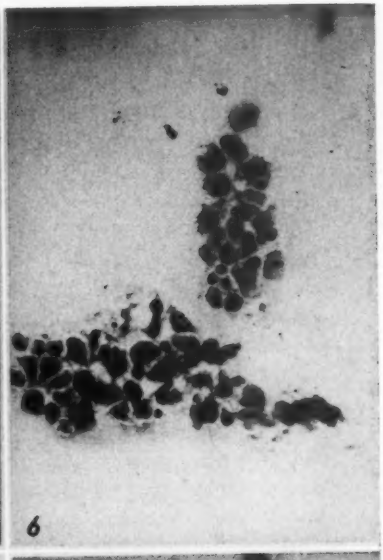
FIG. 5. Margin of a living epithelial sheet from a culture similar to the preparation shown in figures 3 and 4 but photographed with an 8 mm. dark phase contrast objective (AO) showing membrane activity at the free edge of the cells. Magnification 250x.

FIGS. 6–8. Nipple secretion from a Boston Bull bitch. All at 250x. Stained with Jacobson's method.

FIG. 6. Individual cells aggregated in secretion fluid. Monocytes, macrophages and epithelial cells were commonly associated together.

FIG. 7. Direct initial smear showing clusters of small and large epithelial cells characteristic for the secretion from this nipple.

FIG. 8. Epithelial island fixed on the 7th day of culture showing flat expansion of cells characteristic of living elements *in vitro*.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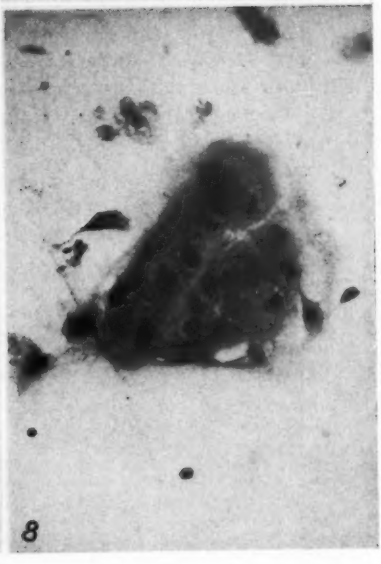
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led to the suggestion that exfoliated cells from various sites of the body might lead to more dynamic studies than those possible with the use of the simple direct smear technique.

As a result of important findings on the diagnostic and prognostic value of cytological studies upon cells in nipple secretions by Jackson, Todd and Gorsuch (1951), a program of investigations in progress is designed to observe the behavior of cells from nipple secretion in tissue cultures.

Since the breasts of aged female dogs are prone to show a wide variety of pathological changes, the secretions of such animals were suspected of providing an ideal experimental material. Initial results are presented in figures 6–11 from preliminary studies on the establishment of growth patterns of exfoliated cells *in vitro* from a Boston bull bitch. Direct smears of a nipple secretion showed isolated tissue cells, leucocytes and macrophages (fig. 6) as well as clusters of cells of various sizes (fig. 7). Tissue culture preparations incubated for 7 days and photographed at the same magnification showed numerous epithelial islands (fig. 8). Material expressed from another nipple (fig. 9) gave rise to sheets of epithelium, often nearly 1 mm. in diameter (fig. 10). In some areas of such colonies the cells had become broadly extended (fig. 11) while the center of the colony showed closely packed and smaller elements.

Epithelial cultures also have been obtained from material expressed from the nipple of human subjects with papillomatous growths. An illustration of such a culture is shown in

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PLATE 3

FIGS. 9–12. From the same dog as that described in figures 6 to 8 but secretion obtained from another nipple. Jacobson's stain.

FIG. 9. Typical ropy clusters of deeply basophilic cells seen in the direct initial smear. Magnification 250x.

FIG. 10. Epithelial island established *in vitro* after 7 days of incubation. Central cells were closely packed and small, while those nearer the periphery were broadly extended as seen in figure 11. Magnification 37x.

FIG. 11. Same as figure 10 but from the area of large cells photographed at the same magnification as those from the initial smear (250x).

FIG. 12. Epithelial cluster established *in vitro* after 7 days from secretion expressed from the nipple of a white female (M.M., 3/17/51, Nix H.) with a diagnosis of intraductal papilloma. Jacobson's stain. 250x.

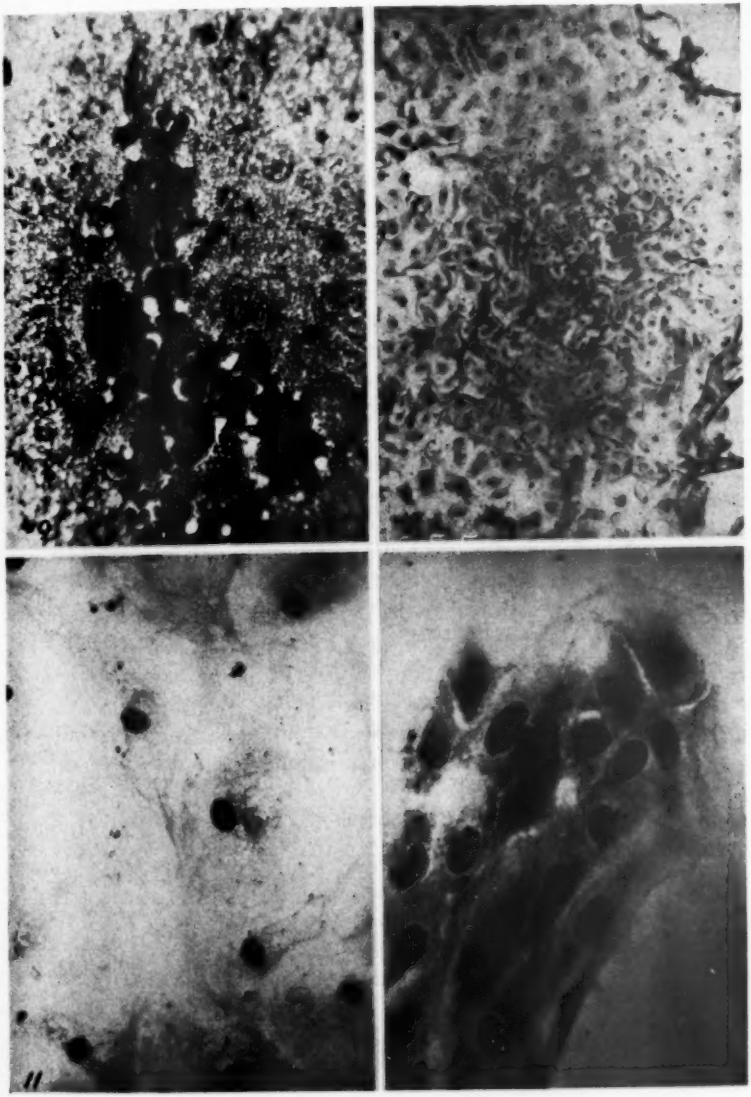

figure 12 but discussion on this subject is reserved until a larger body of data has been accumulated.

Normal Breast Tissue

An understanding of the behavior of normal and pathological breast tissue *in vitro* is considered an essential basis for the interpretation of the type of growth which may be obtained from isolated exfoliated cells.

The activities at the margin of a sheet of epithelial cells (fig. 13) from an explant of human breast tissue which appeared normal in the histological preparation have been recorded in a sequence consisting of more than 300 feet of moving picture film taken at the rate of 8 frames per minute. This film shows various cellular movements including pinocytosis, backward and forward movements of the cellular margin and the behavior of overlapping sheets of cells. Typical epithelial mitosis as well as abortive and endoplastic mitotic patterns can be seen. The analysis of such records forms an essential base line for the interpretation of cinematographic observations on pathological material.

Scirrhous CA of the Breast

Work in progress includes tissue cultures of various types of pathological breast tissue. By way of illustrating results being accumulated under the heading of this category, figures 14–16 represent observations on a scirrhous carcinoma of the human breast.

A photomicrograph of the histological section is shown in figure 14, while the outgrowth on the 12th day of culture is

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PLATE 4

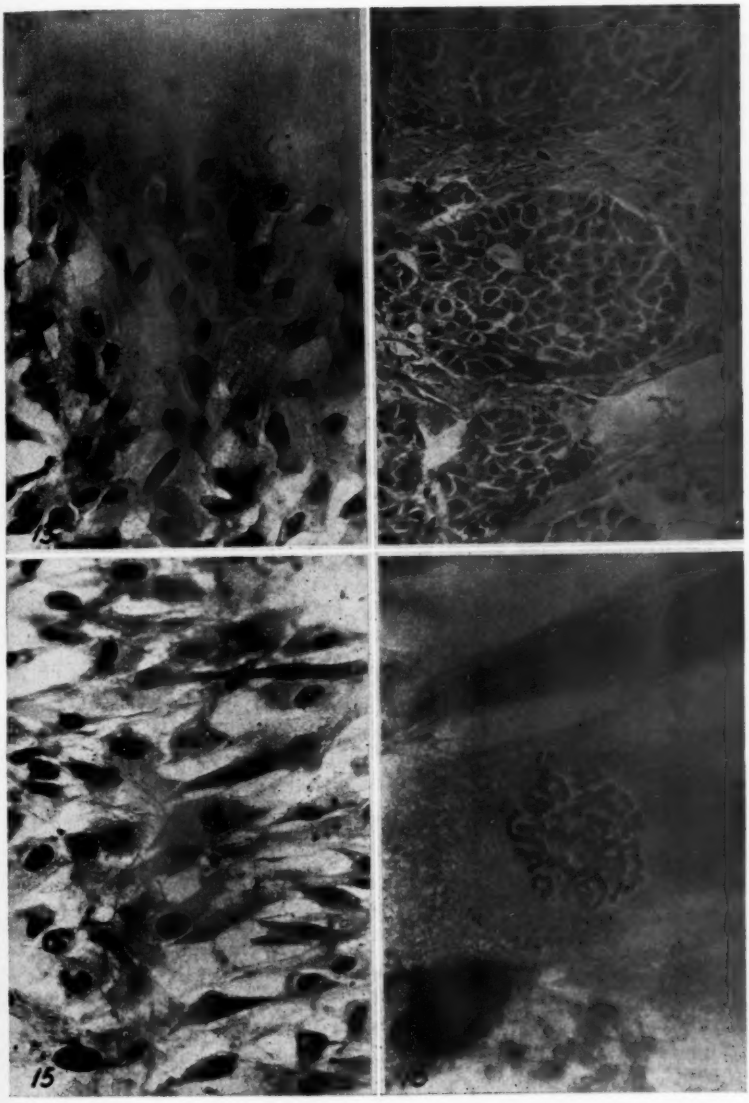
FIG. 13. Normal breast in tissue culture. Patient B.J.D. JSH #11682-G. Age 26, white female. Margin of outgrowth after 13 days of incubation. 250x.

FIGS. 14–16. Scirrhous Carcinoma of the breast (Grade IV). Patient G.D. JSH #12138-G. Age 44, negro female.

FIG. 14. Histological preparation at approximately 250x.

FIG. 15. Outgrowth of spindle cells after 12 days *in vitro*. 250x.

FIG. 16. Mitotic pattern of cell in center of field shown in figure 15 but at a magnification of approximately 1000x.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seen in figure 15. The mitosis in the center of this preparation as shown at higher magnification is represented in figure 16. This serves to illustrate how valuable unsectioned cells growing "on the flat" may be for experimental studies on the chromosomes in neoplastic cells.

Summary and Conclusions

This lecture was designed to call attention to some of the opportunities available for correlation studies on breast tissue in relation to nipple secretion with the use of tissue culture technique.

A brief statement is made regarding methods which have proven useful and a few illustrations have been selected to illustrate typical results.

An intraductal papilloma is shown to have become established *in vitro* as a single layer epithelial sheet whose cytoplasmic features resembled those of the original tissue. Similarity of cellular pathology *in vivo* and *in vitro* appears to be a fairly common event (cf. Gey and Gey, 1936; Rose, Townsend and Pomerat, 1951) and, therefore, important in programs utilizing tissue cultures for diagnostic and experimental studies.

Exfoliated cells collected from the nipple secretion of the dog as well as from the human have been successfully cultivated *in vitro*, suggesting that added information may be obtained regarding breast pathology with the aid of this relatively simple procedure.

It is hoped that information regarding exfoliated cells with direct smear technique may be enriched with *in vitro* methods. Of special interest is the opportunity of studying the effect of additions of chemicals, particularly those of the physiologically active sterol series to medium used in the cultivation of human papillomatous tissue.

By way of establishing reference and correlation data, normal and pathological breast tissue is being grown in roller tubes and various types of records are being made of their structural and behavioral features. Some 400 feet of moving

picture film was presented at the Conference which illustrated cellular movements in cultures of a normal breast and of an intraductal papilloma.

Grateful appreciation is expressed to Mr. George Rose for indispensable assistance with the preparation of cultures and photomicrographs.

BIBLIOGRAPHY

Cone, R. E., C. A. Hooks, C. M. Pomerat, and G. Rose: Correlation studies on prostatic fluid, prostatic tissue and the testis with histopathological, Papanicolaou smear and tissue culture technique. *Texas Rep. Biol. and Med.*, 7:462–467, 1949.

Danes, B.: Pulmonary epithelium of the newt, Triturus viridescens, studied in living cultures with cinematographic apparatus and phase contrast technique. *J. Exp. Zool.*, 112:417–448, 1949.

Gey, G. O., and M. K. Gey: Maintenance of human normal cells and tumor cells in continuous culture. Preliminary report: Cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. *Amer. J. Cancer*, 27:45–76, 1936.

Jackson, D., and A. O. Severance: Cytological study of nipple secretions. An aid in the diagnosis of breast lesions. *Texas State J. Med.*, 41:512–514, 1946.

Jackson, D., D. A. Todd, and P. L. Gorsuch: Study of breast secretion for detection of intramammary pathologic change and of silent papilloma. *J. Internat. College of Surgeons*, 15:552–568, 1951.

Pomerat, C. M.: Tissue Culture Methods. *Methods in Medical Research*, Volume IV, 198–291, 1951. Year Book Publishers, Inc.

Pomerat, C. M., W. W. Nowinski, and G. G. Rose: Tissue cultures of cells from body fluids. *Texas Rep. Biol. and Med.*, 8:521–532, 1950.

Rose, G. G., F. M. Townsend, and C. M. Pomerat: Observations on the cultivation of surgical specimens of human ovaries *in vitro*. *J. Natl. Cancer Inst.*, 2:1223–1257, 1951.

THE POLYSACCHARIDES OF SERUM PROTEINS IN CANCER*

M. R. SHETLAR

From a quantitative viewpoint, the serum polysaccharides represent an important fraction of the blood constituents. Details of their physiological role, metabolism, and chemistry are largely unknown. Biochemically, only one fraction, seromucoid, has been extensively studied. According to Remington (1940), it appears to be composed of an equimolar mixture of acetylglucosamine, mannose, and galactose. Winzler, Devor, Mehl, and Smyth (1949) reported that this fraction is actually a mixture of three components. Their overall ratio of hexose to hexosamine was 15.1:11.9, indicating more hexosamine was present than was indicated by Remington. These investigators also reported the isolation of mucic acid, which indicates the presence of galactose, in their seromucoid preparation. Apparently, mannose derivatives have not been isolated from mucoprotein. However, mannose phenylhydrazone was isolated by Dische (1928) from acid hydrolysates of plasma.

Studies of absorption curves of the reaction mixture resulting from the treatment of serum or plasma proteins with various sugar reagents indicate the presence of mannose and galactose. Shetlar, Foster, and Everett (1948) noted that the tryptophane acid reaction curves with serum were similar to those obtained with an equimolar mixture of glucosamine, mannose, and galactose if an allowance was made for color development of the serum with sulfuric acid not due to the reaction of tryptophane with carbohydrates. Friedman (1949)

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made a study of the absorption curves of the carbohydrates of horse serum proteins with orcinol, carbazole, and skatole and obtained results consistent with an equimolar ratio of mannose and galactose. It appears that the galactose, mannose and glucosamine are definitely present in serum polysaccharides. Other carbohydrates may occur in some of the polysaccharides. Bendich, Kobat, and Bezer (1947) reported the isolation of fucose from hydrolysates of blood group A and O substances.

Lustig and Langer in 1931 demonstrated that the protein-bound sugar of sera from cancer patients was higher than that from normal subjects. Nilsson in 1937 noted an elevated glucosamine content of sera from cancer patients, and Seibert, Atno, and Campbell in 1947 reported elevated polysaccharides in carcinoma, sarcoidosis and tuberculosis.

In order to make a widespread study of these compounds in the sera of a number of patients, it was necessary to develop a fairly rapid analytical method. Merten (1938) estimated serum polysaccharide by determining the difference between free sugar and total reduction after acid hydrolysis of the serum. Lustig and Langer applied the Tillman-Phillipi orcinol method to an alcoholic precipitate of serum. Seibert and Atno (1946) adopted the carbazole reagent of Dische (1927) for a colorimetric determination of polysaccharide in serum. In the presence of the serum protein both the latter reactions compete with the reaction of carbohydrate with tryptophane. Consequently, Sheppard and Everett (1937) suggested that polysaccharide might be analysed colorimetrically in the presence of protein by adding an excess of tryptophane. This reaction was utilized for the development of a rapid quantitative assay of nonglucosamine serum polysaccharides (Shetlar, *et al.*, 1948). The procedure as used at present is as follows:

Reagents: Sulfuric acid solution, 77% by volume. 770 ml. of C.P. concentrated sulfuric acid is added to 230 ml. of distilled water.

Absolute ethanol.

Tryptophane solution, 1%. 1 g of dl-tryptophane (Eastman Kodak) is dissolved in 80 ml. of .075 N_aOH. The solution is made up to 100 ml. with distilled water, filtered, and

stored in a glass-stoppered bottle in the refrigerator until ready for use.

Method: The method for blood serum is as follows: The serum is diluted 1–2 with distilled water. Two-tenths ml. of the diluted serum is pipetted drop by drop into 10 ml. of absolute ethanol in glass-stoppered centrifuge tubes. The precipitate is centrifuged out and washed once with 10 ml. of absolute ethanol. After centrifuging as before, the ethanol is decanted and the precipitate drained by inversion of the tube. One ml. of distilled water and 7 ml. of 77% sulfuric acid are then added, the tube is allowed to stand at room temperature for 10 minutes to dissolve the precipitate, and it is then placed in an ice bath for 15 minutes. One ml. of cold 1% acqueous tryptophane solution is added to each tube without shaking. The contents of the tube are mixed and the tube placed immediately in a boiling water bath for a period of 20 minutes. The tube is shaken once after it has been heated 10 minutes. After removing from the boiling water the tubes are cooled for 5 minutes in an ice bath and then allowed to stand 25 minutes at room temperature. The concentration is determined in a Coleman No. 11 spectophotometer at a wave length of 500 mμ by reading against a blank containing only water, tryptophane and acid, which is run through the above procedure.

Standard: Fifty milligrams of d-galactose and 50 milligrams of d-mannose were dissolved in a liter of solution. One ml. of this solution (100 micrograms of total sugar) was carried through the procedure simultaneously with the protein precipitate.

Results on Cancer Patients

Patients for study were selected from the University Hospitals. Blood samples were taken from patients having a tentative diagnosis of cancer before biopsy and before any treatment was started. Final diagnosis was established in most cases by biopsy.

A summary of results of cancer cases is presented in table 1. In contrast, results of a study conducted on patients who had

TABLE I

Summary of the Serum Levels of Non-glucosamine Polysaccharide, Glucosamine and Total Protein in Malignant Conditions

Diagnosis	No. of Cases	Non-glucosamine Polysaccharide		Glucosamine	Total Protein
		mg. %	% of Protein	mg. %	
Normal Adults	43	111(93–127)	1.59(1.26–2.02)	69(61– 82)	7.00(6.00–7.55)
Ca Skin, Sq. Cell	5	150(123–164)	2.23(1.96–2.53)	88(74–103)	6.74(6.29–7.56)
Ca Skin, Basal	3	132(106–163)	2.10(1.76–2.47)	70(57– 80)	6.25(6.02–6.60)
Ca Lung, Sq. Cell	9	190(148–216)	2.92(2.28–3.36)	112(98–125)	6.52(6.10–7.30)
Ca Stomach, Coll. Cell	9	165(129–189)	2.86(2.32–3.68)	93(82–102)	5.89(4.45–6.88)
Ca Pancreas	6	169(148–184)	3.00(2.45–3.53)	88(74– 96)	5.85(4.44–6.89)
Ca Rectum	7	183(155–240)	3.06(2.47–3.63)	101(78–126)	6.02(5.45–6.92)
Adeno Ca Breast	6	140(120–154)	2.27(2.05–2.47)	73(72– 84)	6.15(5.70–6.44)
Ca Cervix, Sq. Cell	13	165(126–233)	2.57(2.19–3.88)	90(73–119)	6.44(5.25–7.05)
Ca body of Uterus	4	159(131–184)	2.52(2.26–2.92)	85(65–103)	6.54(5.68–7.13)
Ca Prostate	3	178(162–189)	2.80(2.61–2.92)	94(85–100)	6.37(6.30–6.47)
Ca Bladder	2	176(156, 187)	2.53(2.40, 2.66)	94(82, 105)	6.75(6.49, 7.01)
Osteogenic Sarcoma	2	200(144, 256)	2.80(1.95, 3.66)	115(96, 133)	7.19(7.00, 7.38)
Lymphosarcoma	2	234(217, 250)	3.01(2.96, 3.06)	125(114, 136)	7.76(7.33–8.18)
Myle. Leukemia	7	171(119–216)	2.50(1.84–3.38)	96(63–128)	6.96(5.96–8.28)
Lymph. Leukemia	5	161(125–202)	2.85(2.13–3.39)	89(79–108)	5.63(5.12–6.16)
Lymphoblastoma	4	163(138–176)	2.71(2.42–3.31)	97(94–100)	6.06(5.32–6.67)
Hodgkins Disease	3	181(176–189)	3.26(2.93–3.69)	97(94–100)	5.58(5.12–6.02)
Mult. Myeloma	6	200(148–308)	2.44(2.05–3.25)	113(93–177)	7.74(5.88–9.92)
Malignant Melanoma	2	151(150, 152)	2.54(2.47, 2.61)	84(76, 93)	5.95(5.82, 6.08)
Average Malignancies	105	171	2.69	95	6.48
Average Carcinoma	67	167	2.68	92	6.29

benign lesions which were originally suspected of being cancerous is presented in table 2. The only strikingly high result in this group is the one from a patient with hydatidiform mole. A statistical analysis of these data showed that the serum non-glucosamine and glucosamine polysaccharide of the cancerous group were significantly elevated over that of the normal or benign group. However, studies on non-neoplastic pathological conditions indicated that elevations occur in some cases of rheumatoid arthritis, cholelithiasis, ulcerative colitis, nephritis, pemphigus, prostatic hyperplasia, tuberculosis, and in some infections. Essentially normal results were found in sickle cell anemia, pernicious anemia, atherosclerosis, diabetes mellitus, hyperthyroidism, hypothyroidism, hypertension, pituitary insufficiency, multiple sclerosis, and thrombophlebitis.

The serum polysaccharide level varies somewhat in different types of malignancy. Patients having carcinoma of the skin, either basal or squamous cell, as well as those with breast cancer tended to have lower polysaccharide levels than those in which the carcinoma occurred at other sites. Possibly, these two types of lesions are more readily noticed and diagnosed, and consequently not as large when the samples were taken.

The effect of cancer therapy on the serum polysaccharide has not been extensively studied. However, a serial study of the polysaccharide level following the surgical removal of cancer from three patients, one with a carcinoma of the cervix, one with a carcinoma of the lung and one with a carcinoma of the thyroid, are depicted graphically in figure 1. The initially high serum polysaccharide level increased even more in each case a few days post-operatively. This effect is apparently due to the effect of surgery, as sham operated dogs show a similar rise (Shetlar, Bryan, Foster, Shetlar, and Everett, 1949). After about the seventh day the polysaccharide level declined slowly in two cases (Nos. 1, 2). In case No. 2 there was a second transient rise on the 39th day. This elevation occurred five days after initiation of X-ray therapy. This patient's polysaccharide level subsequently fell to normal levels. These two cases have now been followed two and one-half and three years, respectively, and have shown no signs of recurrence. Case No. 3 on the other hand exhibited a drop post-

TABLE II

*Summary of the Serum Levels of Non-glucosamine Polysaccharide, Glucosamine, and Total Protein in the Presence of Benign Lesions**

Diagnosis	No. of Cases	Non-glucosamine Polysaccharide		Glucosamine	Total Protein
		mg. %	% of Protein	mg. %	
Leiomyoma, Uterus	4	134(121–148)	2.04(1.82–2.12)	82(73– 91)	6.58(6.00–7.03)
Polyp, Cervical	1	119	1.90	71	6.26
Endometriosis	8	126(118–133)	1.87(1.67–2.15)	76(67– 92)	6.64(6.00–7.27)
Salpinogitis	2	124(121, 127)	1.97(1.82, 2.12)	76(73, 80)	6.64(6.00, 7.27)
Ruboovarian Abscess	1	127	2.12	73	6.00
Hydrosalpinx	1	104	1.44	69	7.25
Hydatiform Mole	1	203	3.04	102	6.68
Papillary Cystadenoma	1	112	1.67	65	6.73
Adenoma of Breast	2	116(112, 121)	1.90(1.88, 1.93)	70(70, 70)	6.14(5.98, 6.29)
Fibroadenoma Breast	1	150	2.12	84	7.06
Lipoma, Breast	1	113	1.74	69	6.40
Cystic Mastitis	1	98	1.67	68	5.88
Polyp, vocal cord	1	126	1.81	77	6.96
Adenoma, Lung	1	108	1.60	60	6.74
Allergic Bronchitis	1	126	1.92	67	6.57
Adenoma, Thyroid	1	111	1.67	72	6.65
Giant Cell Tumor	1	117	1.89	77	6.18
Average	32	126	1.96	76	6.49

*Conditions in which malignancy was originally considered as a possible diagnosis.

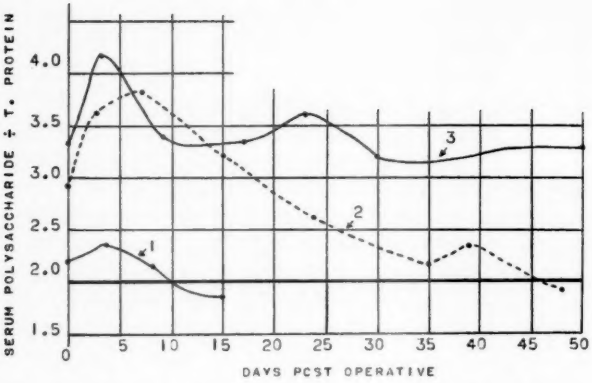

FIG. 1. Effect of Surgical Removal of Cancer on the Serum Polysaccharide Level.

Case 1—Carcinoma of the thyroid.
Case 2—Carcinoma of the cervix.
Case 3—Carcinoma of the lung.

Cases 1 and 2 were a symtomatic 2½ and 3 yrs. after operation respectively.

Case 3—Death occurred outside the hospital on the 60th post-operative day.

operatively, but this was followed by a rise again on the 24th day. The temperature of this patient was elevated on the 21st, 22d, and 23d day and may partly explain this polysaccharide elevation. Subsequently, the serum polysaccharide level again decreased but never decreased to normal levels although he improved clinically. The patient was discharged on the 53d post-operative day in good clinical condition. Death occurred outside the hospital ten days later. Nothing definite can be drawn from such a small study, but there is an indication that serum polysaccharide levels might be of prognostic value.

In order to determine which of the serum protein fractions was responsible for the elevation of serum polysaccharide, the sera from a number of patients with cancer, arthritis, and infections were fractionated by salt precipitation methods (Shetlar, *et al.*, 1950). The polysaccharide content of these fractions was determined by the tryptophane method as described above. The average polysaccharide content of the albumin fraction was significantly increased in the serum from carcinoma

patients and was significantly higher than the average polysaccharide content of the albumin from patients with all other pathological conditions. However, the albumin polysaccharide was also increased to a lesser extent in the benign tumor, arthritis, and bacterial infection group. The polysaccharide of the seromucoid fraction was elevated in cancer patients, but did not account for all of the elevation found in the uncorrected albumin fraction. This indicates that at least one other fraction is affected in the serum of cancer patients. Elevation of this fraction may be directly connected with growth or tissue proliferation, since it is also elevated in the sera of pregnant women as indicated by the data presented in table 3.

TABLE III

Comparison of Albumin and Seromucoid Polysaccharide in Serum of Normal, Fetal, and Pregnant Individuals, and in Cancer Patients

	Adult	Fetal	Pregnant	Carcinoma
Albumin Polysaccharide†	28	17	32	49
Seromucoid Polysaccharide†	12	6	12	21
Albumin Polysaccharide ÷ Albumin*	0.40	0.33	0.70	1.09

†Expressed in milligram percent.
*Albumin polysaccharide corrected for the seromucoid polysaccharide divided by the albumin protein corrected for the seromucoid protein.

Animal Experimentation Undertaken to Clarify the Role of Serum Polysaccharide in Cancer

In an attempt to discover the mechanism by which cancer may cause a rise in serum polysaccharide, various animal experiments were designed. Acute inflammation was studied by injecting turpentine intramuscularly and intrapleurally and by injecting a mixed culture of Staphylococcus aureus, Streptococcus pyogenes and mucin intramuscularly into dogs and then making serial serum studies. All of these types of inflammation resulted in substantially the same response. The serum polysaccharide became elevated, reached a maximum about three to six days after injury, and then decreased. Inducement of granuloma formation by injection intraperitoneally of a suspension of talc, and experimental sham operations on animals resulted in essentially similar effects.

As fever was noted in the dogs with bacterial and turpentine abscesses, and in the animals injected with turpentine intrapleurally, studies were made of the effect of temperature elevation. The temperatures of dogs were elevated by the injection of pyromen* or by simply wrapping the animal in a blanket, tightly muzzling him and heating with a hot pad. An elevation of serum polysaccharide resulted in both cases. When the serum proteins were fractionated as described above, the polysaccharide content of the α-globulin was found to be the only one significantly elevated.

Polysaccharide components of serum fractions were studied in dogs into whom talc suspensions were injected intraperitoneally. The rise in polysaccharide in this case occurred largely in the albumin fraction. Thus it appears that elevation of the polysaccharide of the albumin fraction may be correlated with tissue proliferation.

Further indication that the albumin polysaccharide is correlated in some way with tissue proliferation is furnished by a study of the effect of tumor growth on serum polysaccharide in rat serum (Shetlar, *et al.*, 1950). The albumin polysaccharide was found to parallel rather closely the growth of the Walker 256 tumor in rats. No relationship between tumor growth and the polysaccharide of other fractions was noted, although the polysaccharide content of α-globulin was generally increased in rats with large tumors.

Summary and Conclusions

A summary of the effect of various conditions, in which the total serum polysaccharide is elevated, on the polysaccharide content of the serum protein fractions is given in table 4.

It appears that the polysaccharide content of the albumin fraction is elevated in all cases in which tissue proliferation occurs. This probably indicates that carbohydrate-rich proteins increase in the albumin fraction when growth occurs. Apparently they are not identical with seromucoid. Whenever a process involves fever, the polysaccharide content of the α-globulin fraction increases. If both fever (or perhaps tissue

*A nonprotein bacterial pyrogen obtained from Baxter Laboratories, Inc., Morton Grove, Illinois.

TABLE IV

Summary of the Effect of Various Conditions on the Polysaccharide Content of the Different Serum Fractions

Conditions	Specie	Polysaccharide* Associated with Albumin	α-Globulin	β-Globulin	γ-Globulin
Cancer.....	Human	Elevated	Elevated	Unchanged	Unchanged
Cancer.....	Rats	Elevated	Questionable	.....	.....
Benign Tumor....	Human	Sl. Elevated	Unchanged	Unchanged	Unchanged
Late Pregnancy...	Human	Elevated	Unchanged	Unchanged	Unchanged
Arthritis.....	Human	Elevated	Elevated	Elevated	Unchanged
Viral Infections...	Human	Unchanged	Unchanged	Elevated	Unchanged
Bacterial Infections	Human	Sl. Elevated	Unchanged	Elevated	Unchanged
Artificial Fever....	Human	Unchanged	Elevated	.....	.....
Artificial Fever....	Dog	Unchanged	Elevated	.....	.....
Talc Granuloma...	Dog	Elevated	Questionable	Unchanged	Unchanged
Bacterial Abscess..	Dog	Elevated	Elevated	.....	.....
Turpentine Abscess	Dog	Elevated	Elevated	Unchanged	Unchanged
Surgical Operation.	Dog	Elevated	Unchanged	Questionable	Unchanged

*Polysaccharide associated with a particular fraction divided by the protein associated with the respective fraction.

destruction) and tissue proliferation occur together, the polysaccharide content of both α-globulin and albumin increase. This, in general, is the situation in cancer.

REFERENCES

Bendich, A., E. A. Kobat, and A. E. Bezer: Immunochemical Studies on Blood Groups, V. Further Characterization of Blood Group A and O Substances from Individual Hog Stomachs. *J. Am. Chem. Soc.*, 69: 2163–2167, 1947.

Dische, Z.: Uber Charakteristische Farbreaktionen der Milchsaure, des Methylglyoxals und der Kohlehydrate mit Carbazol und Schuelsaure. *Biochem. Ztschr.*, 189:77–80, 1927.

Dische, Z.: Uber die Natur des Eiweissgebundenen Blut plasmazuckers. *Biochem. Ztschr.*, 201:74–86, 1928.

Friedman, R.: Characterization of Sugar Components of Proteins. *Biochem. J.*, 44:117–126, 1949.

Lustig, B., and A. Langer: The Determination of Free, Protein-bound, and Non-protein-bound Sugar in Normal and Pathological Serum, Cerebrospinal fluid, and Urine. *Biochem. Ztschr.*, 242:320–337, 1931.

Merten, R.: Uber die Bestimmung des gebunden Zuckers in Serum. *Biochem. Ztschr.*, 297:304–314, 1938.

Nilsson, I.: The Glucosamine Content of Blood Serum in Healthy Persons and in Pneumonia Patients. *Biochem. Ztschr.*, 291:254–258, 1937.

Remington, C.: Seromucoid and the Bound Carbohydrate of Serum Proteins. *Biochem. J.*, 34:931–939, 1940.

Seibert, F. B., and J. Atno: Determination of Polysaccharide in Serum. *J. Biol. Chem.*, 163:511–522, 1946.

Seibert, F., M. V. Seibert, A. J. Atno, and H. W. Campbell: Variation in Protein and Polysaccharide Content of Sera in the Chronic Diseases, Tuberculosis, Sarcoidosis, and Carcinoma. *J. Clin. Invest.*, 26:90–102, 1947.

Sheppard, F., and M. R. Everett: Determination of Carbohydrates in Proteins. *J. Biol. Chem.*, 119:1, XXXIX, 1937.

Shetlar, M. R., J. V. Foster, and M. R. Everett: Determination of Serum Polysaccharides by the Tryptophane Reaction. *Proc. Soc. Exp. Biol. Med.*, 67:125–130, 1948.

Shetlar, M. R., R. S. Bryan, J. V. Foster, C. L. Shetlar, and M. R. Everett: Serum Polysaccharide Levels in Experimental Inflammation. *Proc. Soc. Exp. Biol. Med.*, 72:294–296, 1949.

Shetlar, M. R., C. L. Shetlar, V. Richmond and M. R. Everett: The Polysaccharide Content of Serum Fractions in Carcinoma, Arthritis and Infections. *Cancer Res.*, 10,681–683, 1950.

Shetlar, M. R., C. P. Erwin, and M. R. Everett: Serum Polysaccharide Levels in Rats Bearing the Walker 256 Tumor. *Cancer Res.*, 10:445–447, 1950.

Winzler, R. J., A. W. Devor, J. W. Mehl, and I. M. Smyth: Studies on the Mucoproteins of Human Plasma. I. Determination and Isolation. *J. Clin. Invest.*, 27:609–615, 1949.

EXPERIENCES IN SPONTANEOUS REGRESSION OF NEOPLASTIC DISEASE IN MAN*

FRED W. STEWART†

Pathology has never found a definition for cancer. To define requires condensation and simplification of many attributes that contribute to the knowledge of an entity. These attributes expand or contract as data concerning them increase. Until such time when some great generalization becomes possible, the expansion of data seemingly creates spreading of confusion.

Every medical student is furnished with a definition of cancer, usually one that merely emphasizes one supposed attribute of the process modified by one of two adjectives. Thus, Ewing defines a tumor as follows: "A tumor is an autonomous new growth of tissue." This statement appeared in the first edition of *Neoplastic Diseases* in 1918. In the last major textbook of pathology to appear in this country this definition is given again, although very slightly modified: "A neoplasm is an uncontrolled new growth of tissue." These two definitions vary in the matter of two adjectives—"autonomous" and "uncontrolled." Autonomous means existing independently or responding or reacting independently of the whole. In the field of biology there is no such thing as autonomy, and "uncontrolledness"—if I may coin a word—is an equally inacceptable concept. Because biology has failed to discover laws of tumor control, it has no right to assume that they do not exist. Actually, investigative medicine is forced to admit that they *do* exist; for if they did not, cancer research in turn *should not* exist, because it could have no future. Thus, the mere existence of research constitutes a denial of the definition of cancer.

Better than the words "autonomous" and "uncontrolled" might be the adjective *heterotopic*, which refers to *the ability*

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†Pathologist, Memorial Center for Cancer and Allied Diseases, 444 East 68th Street, New York 21, New York.

acquired by the tumor cell to exist outside of its locus of origin. The skeptic would of course say immediately: "How about carcinoma in situ, where the tumor cell is still within—let us say—the mucous membrane of origin?" This is not really an objection, because even while within the germinal layer of the epithelium of origin, the tumor cell looks like a cell apart. Further, as it moves toward the surface it becomes heterotopic, because the immature-looking tumor cell is heterotopic as soon as it leaves the environment of the germinal layer. It is obviously heterotopic *within the tissue of origin* as soon as it leaves the immediate environment of the cells of origin, whether or not it remains within the organ. Be that as it may, little satisfaction can be obtained by quibbling about definitions for actually there is no definition of cancer that cannot be attacked.

Carcinoma In Situ

By definition, a carcinoma in situ is a neoplastic entity wherein the tumor cells still lie within the epithelium of origin. We accept the fact that they are cancer cells because, when they leave this site of origin and infiltrate or disseminate, their appearance is that of the cells still resident within the site of origin. This similarity is the only thing we have to identify them as cancer cells. Hence, in our present state of knowledge, they *are* cancer cells.

There is abundant evidence that these cancer cells may have remained within this site of origin for ten years or longer. This extensive period of relative quiescence, although most commonly recognized in the uterine cervix, is by no means thus restricted. It can be observed in in-situ malignant cervical lesions, laryngeal cancers, glandular cancers of the endometrium, lobular carcinomas of the breast, and perhaps other neoplasms. Chronology can be established only by fortunate accident in such cases. Thus, in a biopsy report of in-situ cancer of the larynx occurring many years ago the clinician doubted the report of carcinoma and did nothing. Infiltrating carcinoma first became apparent seven years later.

Recently, a patient was admitted to a certain clinic under the diagnosis of mammary carcinoma arising in the scar of

excision of a benign lesion. The benign lesion had been removed a decade before. The infiltrating cancer was of the lobular pattern. Fortunately, the sections of the old benign lesion were available for re-examination, which revealed lobular carcinoma.

The chronology of the endometrial cancers was well established by Hertig. He re-examined curetted materials taken at various intervals prior to the curettage that demonstrated unmistakable cancer. I do not wish to infer that this slow evolution is a feature of all cancer development; if such were the case, we would have no means of proving it. Rather I suspect it to be a feature of *some* examples of many types of cancer, and a feature common rather than rare. I do suggest that slow evolution is evidence of control.

Every cancer must begin as a cancer in-situ—although this term is popularly applied to one anatomic type, cancer of the cervix. It may be equally applied to many mammary cancers, not alone the in-situ lobular carcinomas, but also the papillary cancers of ducts that fail to infiltrate. The term is suited to describe certain bronchus cancers, many early cancers in rectal polyps, some gastric cancers, some endometrial growths, and would have a much wider application if we only saw more early lesions.

In these in-situ stages there *is* evidence of cancer control. In the epidermoid cancer the tumor cells respect the basement membrane; instead of infiltrating and destroying it they move outward toward the layer that normally desquamates—thus following the course normally assumed by the noncancerous cell. When the tumor cells extend inward, they first fail to infiltrate, and only progressively replace the linings of preexisting gland ducts. In other words they exhibit a certain property of tissue interrelationship that fails to depart in any essential from that exhibited by the normal cell.

In an in-situ gastric cancer, the cancer cell seems liable to necrosis and erosion; but the regenerating cancer cells, instead of infiltrating, regenerate to replace the lost surface. Therefore, the in-situ gastric cancer may be confused clinically with a peptic ulcer, because it heals over eroded areas under dietary regime and thereby lends a false sense of security. Thus, the

normal attribute of healing effort is shown—a sort of normal physiologic behavior, rather than the much touted "autonomy" and "uncontrolledness." Later on, when some attribute or attributes are added to behavior, more characteristic features of neoplasia appear, rendering the totally unbiological word "abnormal" more appropriate.

In some instances of in-situ epidermoid carcinoma of the uterine cervix, positive smears have been obtained repeatedly. Then, quite suddenly the tumor cells cease to appear. If we assume such cases do represent early cancer—and we are relatively certain they do—then we must admit that the normal process of desquamation rids the mucosa of its tumor. Further, the stimulus to further production of tumor cells ceases. In but one instance have I had reason to believe that a fully developed cervix cancer had disappeared. This was in 1946 in a patient with incontrovertible biopsy proof of cervical carcinoma, which had developed beyond the simple in-situ stage. This patient has no cancer today. Furthermore, she has had no treatment unless it be said that the securing of a very small biopsy not more than 2 mm. across had removed the cancer. This idea is conceivable but extremely improbable.

There are curious features about these in-situ cervix cancers in Jewish women that interest me greatly. At my request, our statistics department investigated the incidence of cervix cancer in a 4-year period. There were 702 cases and of these but 26 were in Jewish women (3.7%). The total hospital population is at least one third Jewish. In the detection clinic however about one fourth of the carcinomas in-situ were in Jewish women. Yet, the frequency of these tumors in all women examined was the same as known incidence rates of cervical carcinoma. Unless there is some discrepancy in the data, which of course there may be, the impression is that such lesions in Jewish women disappear. Or it might be that their total evolution is so slow that they do not contribute their full bearing on the frequency of developed cervix cancer. Are we all wrong in regarding these in-situ lesions as cancers? This I doubt. Are there racial behavioristics? This one must question because there is no such thing any more as a Jewish

race. Thus, the matter remains unanswered. More data are required with larger figures.

In the whole question of the significance of early alterations that can be interpreted as in-situ cancer of the cervix, we may be overcalling our shots. This overcalling is inevitable because of simple enthusiasm. When one has to his satisfaction identified a very early stage of a process that deserves the name cancer, he cannot help trying to identify an even earlier phase. In so trying he steps into a shadowy zone where his judgment leads him astray. His probabilities are apt to become his certainties, until he has what may be a rude awakening.

Thus I believe that the diagnosis of in-situ cancer of the cervix is made too frequently. There are diskaryoses to be seen in pregnancy and in trichomonas vaginitis that are very confusing and are being called cancer. I do not think pathologists are well aware of this pitfall.

Recently, I was talking with Dr. TeLinde, chief of gynecology at Hopkins. I asked him about the diagnosis of in-situ cancer of the cervix in pregnancy, and he said he did not dare make it. I asked him what he would call the same type of cytologic change in a middle-aged nonpregnant woman, and he said he would call it in-situ carcinoma. I am in complete accord with this point of view, but it opens more problems than it solves. Why are two lesions essentially identical in pattern different in different physiologic states? Are they both cancers? Do they represent processes that are usually considered irreversible; and *is* one reversible, though cancerous, by "normal" means? In other words is it spontaneously controllable? If it is, then have we not a lead toward the controllability of the other process that does not occur in pregnancy? Have we a lead on causation? Or, as a let down, will we on closer study be able to separate the two lesions and recognize some differences that force us to withdraw one from the cancer category?

Spontaneous Control

Spontaneous or *induced* cancer control is evidenced in certain tissue reactions of the host. In one variety of cancer of

the breast a tremendous lymphoid infiltrate occurs. In fact, for this type we have invented the term "medullary carcinoma with lymphoid infiltrate." The prognosis with this type of cancer is much better than with any other type of infiltrating mammary carcinoma, even though nodes are involved. We believe this massive infiltrate indicative of a state of spontaneous host resistance. As an example of what we might call *induced* resistance, I refer you to certain observations we have made on cervix cancer treated by external radiation via vaginal cones. When a patient has a cancer of the cervix that is partly in-situ and partly infiltrative and when one treats this patient by X-ray through vaginal cone and studies the result pathologically, it will be seen that the in-situ portion will persist whereas the adjacent superficially infiltrative portion will show very advanced degenerative changes directed toward disappearance. Since differences in dosage cannot be significant within areas of no more than one or two millimeters, we have to assume that the difference of behavior is due to the fact that the cancer that has infiltrated is in the presence of a *reactive* bed. Further, we assume that the effect of the radiation has been sufficient on the tumor cells so that the forces of the reactive bed, whatever they are, become manifested. Thus the body is *not* accepting the tumor cell passively, at least in this stage. Unfortunately the other extreme also exists in the cancer field where the acme of tumor acceptance is seen. This is best illustrated in advanced disease where, as in a recent case reported by Dargeon *et al.*, transfer of melanoma occurred from mother to fetus, resulting in death of the infant in early life.

The Influence of Hormones

The field of prostatic cancer is curiously contradictory. In the last decade or so it has been widely admitted that some 25% or more elderly subjects had miniature prostatic cancers. Since it is apparent that these have not behaved like clinical cancers, one is justified in assuming that they are restrained by some sort of physiologic control for long periods. We have no knowledge of wherein this control lies. However, not only

in these miniature cancers but also in clinically fully developed prostatic cancer, I have sometimes noted cytologic patterns suggestive of those seen in the prostates of therapeutically castrated and estrogen treated individuals. Therefore, it may well be that spontaneous alterations of hormone environment may be invoked in explaining this long evolution or restrained activity.

This attitude is reinforced when one examines the incidence curve of clinically detected breast cancer. This curve shows a double peak. It is rising prior to the menopause, then it falls, and then it rises again. This information, coupled with the knowledge that certain mammary cancers are favorably influenced by an altered hormone environment, suggests that the menopause provides a changed hormonal environment unfavorable to the progress of certain mammary cancers. Therefore, the clinical appearance of some breast cancers is slowed. Had no change in environment occurred, the lesions would have progressed at a normal rate and eliminated the slump in the incidence curve.

We know that prostate cancer exhibits regressive alterations after castration or hormonal therapy and that breast cancer also does. Therefore, remembering the long latency periods sometimes involved in these diseases, may we not be justified in believing that some lesions in these organs, which ordinarily would progress, may involute completely? We have no evidence from the prostate that such is so. In the breast, however, it is quite certain that papillary lesions of ducts which are capable of giving rise to breast cancer do show, at times, very pronounced involutional patterns tending toward atrophy and fibrosis. The fact that some would deny that such lesions can pass over into cancer does not disturb me, because no one who has seen a large amount of breast material would deny that they do. Then if we admit that there are levels in the development of these lesions where one could not be certain whether the cells one sees are cancerous or not, is not one by inference justified in assuming that some small early breast cancers disappear spontaneously? This evidence, in turn, would seem to indicate that we are witnessing control.

Embryonic Controls

It may be said that these are specific examples and that such phenomena cannot exist in other parts of the cancer field. However, no one knows that such is a fact. Because we do not know of controls, we have no right to assume their nonexistence. We see evidences of control only in those situations where we have learned something about the modifications of environment possible. Our status is like that of embryology prior to Spemann's classic demonstration of organizers. Certain patterns of tumor growth are close duplications of stages of embryonic development, developmental patterns that mature under control. Let me cite for you an example. Two microscopically similar tumors exist, one in the testis and one in the ovary. The first is commonly called seminoma, the second disgerminoma. Some months ago I received a section of ovary from a fetus. I looked at it and said it was a prenatal disgerminoma. Another pathologist, who had not forgotten his embryology, said the material was only cords of early sex cells. After he said that, I secured a number of ovaries from embryos. Sure enough, at a certain embryonic age I could have thought that every ovary contained disgerminoma. Yet, naturally they couldn't have been tumors. Instead, they would have undergone normal maturation as they fitted into the pattern of organization of the host. Still, seen in a young adult, they would have constituted a tumor.

Just what is a placenta? In certain stages an early placenta looks like a malignant choriocarcinoma. Furthermore, it acts like a cancer. It invades and destroys the uterine wall and derives its blood supply from curious lakes of blood without formed vessels. Its cells are large and atypical. Remnants of placental cells break off and exist temporarily in such organs as the lungs, and then they regress. Sometimes, as in the case of what one calls chorioadenoma destruens, the process becomes even more cancerlike in clinical behavior, only to regress. The diagnosis is exceedingly difficult for the pathologist, and obviously many cases of cured choriocarcinoma reported were never real choriocarcinomas at all. Every *early* placenta looks and for a period acts like a cancer. I like to

think of it as a cancer, in the high proportion of cases under control. If we don't think of it as a cancer, we have to abandon the microscopic criteria for the diagnosis of cancer—something I am not willing to do.

Spontaneous Regressions

Striking evidence for the state of control appear in the admittedly rare instances of spontaneous regressions of established cancer with proved metastases. I say "spontaneous" although naturally these instances are not examples of tumor disappearance in the absence of *all* treatment, but involve therapeutic procedures either insufficient to cure disease or not even directed to the site of disease. I wish to give you examples. In all but one case I have myself seen the pathological material. In the instance where I did not see it, the exploratory surgery was performed by our clinical director, Dr. Allen O. Whipple while at the Columbia-Presbyterian Medical Center. The pathologic study of this case was made at Presbyterian.

The first case I saw was one of uterine myosarcoma treated by Dr. George Pack. The tumor was wholly inoperable, being spread throughout the pelvis and in the mesenteries. The lesion was soft, very vascular, and hemorrhagic. After biopsy was performed, the patient was treated by a radium bomb. As might have been expected, nothing happened. There was no evidence of any radiosensitivity, and the mass failed to regress at all. Then just before the completion of treatment and within the course of almost hours, a dramatic change occurred. The patient developed a high fever, an urticarial rash, a high eosinophilia, and within a few days lost kilos of tumor and ascitic fluid. The tumor completely disappeared; and when last I heard at least 10 years later, the patient was well. Five years or so after treatment she had some insignificant lesion of the cervix for which she received a small amount of radium. She repeated the hypersensitive reaction. What I assume happened was that some alteration occurred in the tumor protein of this patient and she became sensitized to her own protein. This, in turn, provoked an intense immune reaction. Certainly this cancer came under biologic "control."

The next case was in an infant a few months old. He was brought to the hospital with multiple tiny subcutaneous nodules. When one was subjected to biopsy, a diagnosis of neuroblastoma was made. After the final result I sent these sections to various pathologists in New York and Boston and always got back the same diagnosis: neuroblastoma. The child had small radium plaques applied to one or two of the nodules to see if they exhibited enough sensitivity to warrant, let us say, general body irradiation. They did not. He also had a little of the old Coley toxin—not really treatment. After being discharged with a hopeless prognosis, the nodules proceeded to disappear. Today, more than five years later, he is a perfectly healthy child. Here no known allergic reaction took place.

Back in 1937, I saw another case of metastatic neuroblastoma in a young infant, this time producing a destructive metastasis in the upper end of the left femur. We have to say it was a metastasis, because we have no reason to expect a neuroblastic tumor primary in a bone of the lower extremity. This child was treated by X-ray directed in adequate doses to the involved femur. Treatment was directed to the metastases and not the primary, which probably was retroperitoneal if it was in the usual spot for these tumors. This child is now 14 years old and has no trace of tumor. The primary must have been under control, perhaps through some spontaneous maturation—such as was described years before by the late Harvey Cushing in a slightly similar situation. Only two months ago I received follow-up data on this case. Again I examined the biopsy material and could arrive at no other diagnosis than that originally rendered.

At this same time, I saw the initial X-rays on this last case and took them to our department of roentgenology. There, I was happy to receive uncoached a report that the lesion was consistent with metastatic neuroblastoma. The doubter will of course say perhaps these were not tumors. Perhaps embryonic neuroblasts disseminate to such sites as skin and bone and vanish in the course of normal maturation. However, I have to say that these neuroblasts look microscopically like

tumor; they grow and destroy; they are heterotopic in location; and hence they fulfil the *definition* of cancer. Wyatt and Farber describe a cure of neuroblastoma differentiating into its adult counterpart after incomplete surgery.

I am indebted to Dr. John Godwin of the Ochsner Clinic for still another example of extreme regression of neuroblastoma in infancy. At birth the infant's abdomen was enlarged. No increase in this enlargement was observed by the parent during the first month of life. But at the end of that month, there were noted multiple subcutaneous nodules 2 to 4 cm. in diameter scattered over the abdomen, groins, back, and head. These were large enough to produce bluish discoloration of the skin. When admitted to the Clinic, the cutaneous nodules were noted, and in addition the abdomen was markedly protuberant and tense. A large mass occupied the region of costal margin to iliac crest on the right. It extended 5 cm. over toward the opposite side at the level of the umbilicus. Excretory urograms showed both kidneys displaced downward, and the right pushed medially until its ureter lay in the midline of the abdomen. Biopsy of the cutaneous nodules (2) showed neuroblastoma, which diagnosis I have confirmed beyond any question. The only treatment administered was a little nitrogen mustard over a 4-day period, and this had no immediate effect whatever during a month of observation. Then, 6 weeks after discharge, the abdomen began to shrink and the cutaneous lesions started to disappear. On readmission to the clinic about 7 months after first admission, the child appeared like a healthy infant. Only two cutaneous nodules could be located. The liver edge was palpable 3 fingers below the right costal margin, which does not indicate any great enlargement. Excretory urograms showed the kidneys still low, but the previously displaced right kidney in a much more normal position. The original mass was much smaller. I do not maintain that this child will be cured, and in fact just yesterday learned that new cutaneous nodules have appeared. However, the fact that this mass regressed enormously late after treatment and in a fashion

that could not well be related chronologically to treatment reinforces my belief that these other cases are true examples of spontaneous regression of neuroblastoma.

And while thinking of tumors of children it may not be impertinent to recall the bulky hypertrophic angiomas of infancy, ofter miscalled sarcomas by the uninitiated. These tumors used to be treated vigorously by X-ray, radium, sclerosing agents, before it was realized that their tendency was to spontaneous disappearance, the local circulatory apparatus becoming "organized."

No clinician who treats many examples of acute leukemia—usually defined as a malignant tumor of the marrow—is without these curious examples of profound remissions of the disease. When this happens, the peripheral blood returns essentially to normal status, sometimes for a considerable period.

Recently, I saw a node from a patient who had Hodgkin's disease. Over a decade before Dr. Roy Kracke, one of the best known American haematologists, had made a diagnosis of leukemia on this patient, but the leukemia had vanished. Perhaps it was infectious mononucleosis, and unfortunately Kracke is not alive to defend his diagnosis; but I do not see reasons to doubt him.

To one who witnesses the temporary disappearance of large leukemic masses under ACTH, transient though these disappearances are, "control" is an obvious feature of this tumor. Only the why and the how are great problems. In certain examples of lymphosarcoma, a history is obtained showing repeated enlargement and regression of nodes prior to the establishment of clinical stability of the disease. Unfortunately, we do not know just what such nodes show pathologically—that is, whether they are at that stage lymphomatous.

The case of controlled cancer observed by Dr. Whipple was in a nurse. She was explored and found to have a large, inoperable malignant hepatoma. Instead of dying of the disease in a relatively brief period, she survived in apparent health for some twelve years finally dying an accidental death. Autopsy revealed a small shrunken lesion completely fibrotic and with only traces of atrophied tumor cells.

In the old days, there were a few strange events in patients treated with the old Coley toxins. It is generally presumed that the effect of toxins was from hemorrhages induced in the tumors. Yet, I seriously question whether multiple hemorrhages can themselves be responsible for the disappearance of large masses of involvement, even though one might invoke this explanation for the destruction of small microscopic deposits. Let me cite an example. A man had amputation of a lower extremity through the lower femur for primary reticulum cell sarcoma of bone. Disease recurred the entire length of the remaining femur. A metastasis occurred in the skin of the abdominal wall. This was excised and its character proven. All disease vanished under Coley toxin alone and the patient is still well now after 30 years. I don't think the result was from hemorrhages in the tumor. Hemorrhages always leave peripheral viable tumor cells. The only exception to this rule is the very rare minute testis tumor that produces metastases, but where the primary through spontaneous hemorrhage is reduced to a small scar without residue. This occurs in testicular choriocarcinoma and in seminoma, although in the former a residue of nonmalignant teratoid element may persist. In the Coley toxin cases, I like to think that some combination of toxin and tumor protein has occurred and made the tumor antigenic, but naturally one lacks all proof.

The whole field of metastatic cancer is riddled with examples of growth control. Every late metastasis is an example of control. When a successfully resected rectal cancer reappears, let us say, in the thyroid as I have seen it do more than once after as long as seven years, or when a breast tumor or a melanoma first exhibit metastases after 20 years, are we not justified in assuming a long interval of control of growth? If we knew wherein this control existed, we would have gone a long way toward the answer to cancer.

One of the most striking examples of what we may call latent metastasis appeared quite recently. The patient was a woman aged 55. She first entered Memorial complaining of shortness of breath. She expired 11 months later from the effect of her tumor. The point is that this tumor was a type

of ovarian cancer, and the involved ovary had been removed 35 years before her first admission to Memorial. Other examples of long latency with this type of tumor exists, but this is the longest to my knowledge. At autopsy there were good and sufficient reasons histologically for this long course. The tumor varied greatly from area to area—in some areas being actively proliferating, in others atrophic-looking, degenerate, sclerotic, and with the calcific deposits that commonly characterize the lesions naked, without their cuffs of tumor cells. To be sure this tumor was growing. However, at the same time it was disappearing, even though the ultimately attained balance was in favor of the tumor rather than the patient. A similar case at the New York Hospital had a duration of 33 years.

I suppose the all-time record for behavior of cancer is to be found in a case of cylindroma—or if you prefer the term, adenoid cystic carcinoma—of the salivary gland that was first noted at the age of 15, was first operated at the age of 29, and killed at the age of 80.

Thinking in the cancer field is perhaps too largely directed to methods of artificial destruction of the cancer cell—either by its radical removal or its chemical destruction. There has not been enough thought given to *biological control* by the host. Of course the former is easy when the setting is favorable, and we lack all the knowledge to undertake the latter. Still, I am willing to predict that the solution will be the latter, and that it may not be too many decades away.

In closing, I wish to emphasize that the cancer cell, though superficially anarchical, *is* not, nor *can* it be unbiological, just so long as it is part of a biologic host. Moreover, there are differences in the patterns of host acceptance amounting to evidence for host control. Therefore, one may not be too far out of line in suggesting that great profit may ensue by redirecting some of the efforts at destruction of tumor cells or their radical removal toward an understanding of host-tumor interrelation, an understanding that must precede any biologically-planned attack on the cancer problem. One's philosophy toward the cancer problem may be engendered by

one's attitude; to him who sees mainly anarchy, the problem's outlook is surely bleak. But where one looks more optimistically and sees evidences to justify optimism by the absence of this much touted "unbiologic" anarchy, then surely the ground for optimism has been attained.

REFERENCES

1. Dargeon, Harold W., Joseph W. Eversole, and Vincent Del Duca: Malignant Melanoma in an Infant. *Cancer*, 3:299–306, 1950.
2. Wyatt, George M., and Sidney Farber: Neuroblastoma Sympatheticum. *Am. J. Roentgenol.*, 46:485–496, 1941.
3. Wolbach, S. B., and H. Cushing: Transformation of Malignant Paravertebral Sympathicoblastoma into Benign Ganglioneuroma. *Am. J. Path.*, 3:203–216, 1927.

CANCER AS A PROBLEM OF PROTEIN RESEARCH: RECENT TRENDS*

G. Toennies

The specific chemistry of cancer tissue is still largely unknown territory, although exploratory expeditions can register many recent gains. Before reviewing some of the trends of exploration in this unknown territory, I should like to consider a little of the known characteristics of the phenomenon cancer.

Figure 1 shows, for persons of different age, the statistical frequency of occurrence of two phenomena: death (Dublin, 1942) and cancer (Lindsey and Cohart, 1950). Death occurs 5 to 10 times as often as cancer, but both frequencies increase in a very regular fashion with age, according to the law of compound interest and with a similar interest rate of 8 to 9 per cent per year. You see, incidentally, that while the average life span has been increased through lowering of mortality in the younger ages, the rate of aging, as defined by the rate of increase of the probability of death, has not changed a bit. The full lines in the graph represent actual data, while the dotted lines are extrapolated conjecture. Are these two similar rates of increase causally related? Perhaps it is worth remembering that the one factor which in animal experimentation has consistently lowered the incidence of spontaneous cancer is caloric restriction (Tannenbaum, 1942) and that this is the same factor which has also slowed the rate of aging, by increasing the normal life span of rats (McCay, 1942). Also, life insurance statistics say that among over-weight people the frequency of cancer is higher (Tannenbaum, 1940), as well as the over-all incidence of death (Dublin, 1942). Another piece of evidence for the close relation between aging and cancer is the observation (Dunning and Curtis, 1946) that the percentage of miscellaneous neoplasms

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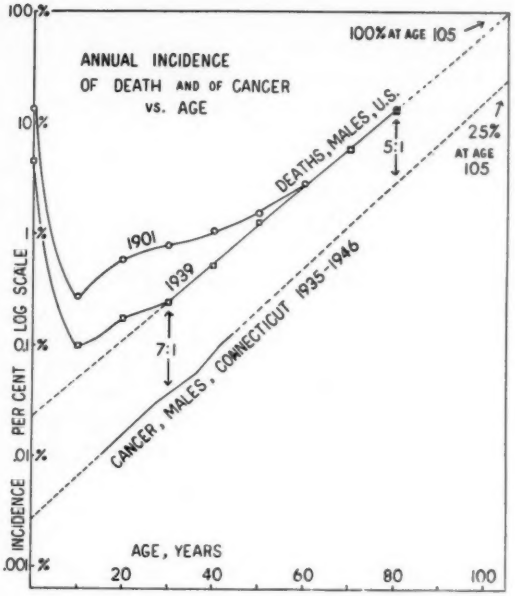

FIG. 1. Annual incidence of death and of cancer in American males, at different ages. Graph constructed from data of Dublin (1942) and Lindsey and Cohart (1950).

occurring in groups of rats of different strains is proportional to the average life spans of the groups. And again, it has been demonstrated recently that caloric restriction lowers mitotic frequency (Bullough and Eisa, 1950) in a similar pattern as it lowers the frequency of cancer.

As you can see, both of the frequencies shown on the graph increase in a self-accelerating fashion, that is, to the chemist, in the way of an autocatalytic reaction. It is not enough, then, to assume that each cell division involves a certain risk of cancer genesis, but one might be inclined to assume that the susceptibility of the cell to cancer increases with each successive generation of cells. In this light the observation of malignant transformations on long-continued tissue culture of normal cells (Gey, 1941, and Earle, 1943–44) seems almost

natural. And in this light the problem of the biochemistry of cancer may be the same as the problem of the biochemistry of death, with other words the problem of aging itself.

What I am suggesting is probably nothing new. It is this. Let us view the cancer problem under three headings:

(a) What is it that increases the susceptibility to cancer with age? This may be the problem of cellular aging.

(b) What are the distinguishing characteristics of a cell that has become cancerous?

(c) What are the extracellular stimuli that initiate the transformation?

All three approaches offer hope for the defeat of cancer. I submit that as a result of the discovery of chemical carcinogenesis some 30 years ago the accent of chemical cancer research has been on the third approach. The second approach, that of characterizing the cancer cell, has been largely dominated, as far as chemistry is concerned, by Warburg's attack on carbohydrate metabolism about 25 years ago. It now seems to enter new channels, as a result of the recent advances in analytical and metabolic research technics, exemplified by isotopic tracers, microbiological assay, electrophoresis, spectroscopy, etc.

Perhaps it is time for the chemist to consider, more than he has in the past, the first-named approach: the matter of cellular aging.

After this digression, let us now return to the protein problem, in reference to the second approach, namely, the characterization of cancer cells. The search for distinguishing characteristics of cancer tissue in terms of proteins, amino acids, enzymes, nucleic acids, etc., has proved to be an elusive quest. In the face of the accumulating evidence one might be tempted to conclude that the anarchistic and subversive character of the cancer cell is carefully disguised behind a cloak of biochemical respectability. Carbohydrate metabolism in the form of aerobic fermentation was thought to be a distinguishing stigma of the cancer cell. It is now becoming clear—and I am referring here to very recent work (Weinhouse *et al.*, 1950; Wenner et al., 1950) at the Institute for Cancer Research in Philadelphia—that cancer tissue is equipped with all the enzymes and co-

factors required for the performance of the oxidative Krebs cycle, although the amounts of these factors possibly may be suboptimal compared with most normal tissues and compared with the energy demands of the rapidly growing tissue. What is impressive is that the distribution pattern of these particular enzymes tends toward a striking uniformity among different types of neoplasms. This is, of course, by this time a familiar theme. It first came into focus when Dr. Williams here in Texas investigated the vitamin content of different tissues about 10 years ago (Pollock, Taylor, and Williams, 1942), and when Dr. Greenstein explored the enzyme levels (Greenstein, 1940). The same story seems to be told by the striking paper-chromatographic pictures of Dr. Roberts (1949) in St. Louis, of free tissue amino acids: a modest uniform pattern in different kinds of cancer, compared with the individualistic variety among normal tissues. And now the same theme is repeated by the enzymes of carbohydrate and fatty acid metabolism.

Amino acid analyses of cancer proteins and normal proteins, carried out by modern methods, have been very recently reported from several places. As an example I should like to show a chart which I constructed from data of Sauberlich and Baumann (1951) (Fig. 2). Each line represents one amino acid. The end points projected on the abscissa give the range of values found in normal tissues, and the same points projected against the ordinate give the range of values found in a number of malignant tissues. It is evident that differences do exist, but they are of a secondary nature, as it were. Generally, the sequence of relative abundance is the same in both types of tissue. In only one case was there no overlap. In all cancer tissues methionine was consistently lower than in the normal tissues.

The inset shows somewhat similar data (Toennies, Kolb, and Gallant, unpublished data) which we obtained five years ago, but which have not yet been published. Human tissues were fractionated by means of salt solutions. Again there were no drastic differences in the soluble cytoplasmic proteins, although the residual protein suggested some significant differences.

Schweigert and associates (1949) went one step further recently by separating tissue homogenates into morphological

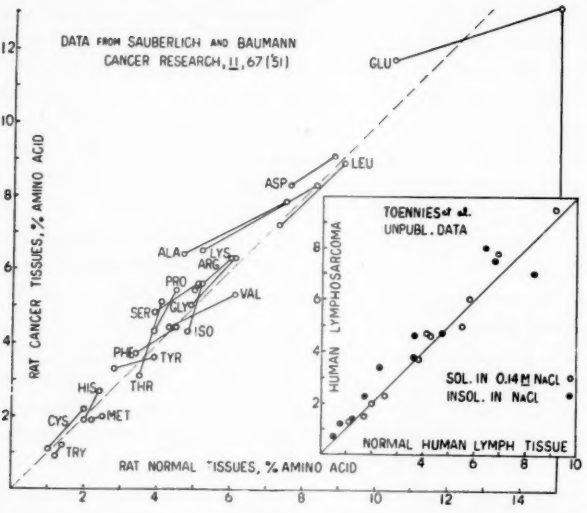

FIG. 2. Amino acid composition of protein of cancer tissues of rats and of normal rat tissues. Graph constructed from data of Sauberlich and Baumann (1951). For each amino acid the end points of the line projected on the abscissa give the range of values found in normal tissues, and the same points projected on the ordinate give the values found in malignant tissues. The inset gives data on the amino acid content of normal and malignant lymph tissue. The tissue proteins were separated into three fractions: those soluble in 0.14 *M* NaCl, those soluble in 1 *M* NaCl (nucleoprotein, analytical data not shown), and those insoluble in NaCl solution.

fractions: nuclei, large granules, small granules, and supernatant material. Their tabulated results seem very interesting. I have taken the liberty of converting some of their data into graphs (Fig. 3). The amino acid analysis of each tissue fraction is shown as a profile. The individual amino acids appear from left to right in the order of average increasing abundance. The scale is logarithmic, so that equal vertical differences represent equal percentage differences. For each morphological fraction, the cancer values are superimposed on those of the corresponding normal tissue. A higher value for the cancer tissue is shown by a black area, a lower value by a shaded area. The over-all pattern again is similar everywhere. However, the data definitely suggest that in general the differences

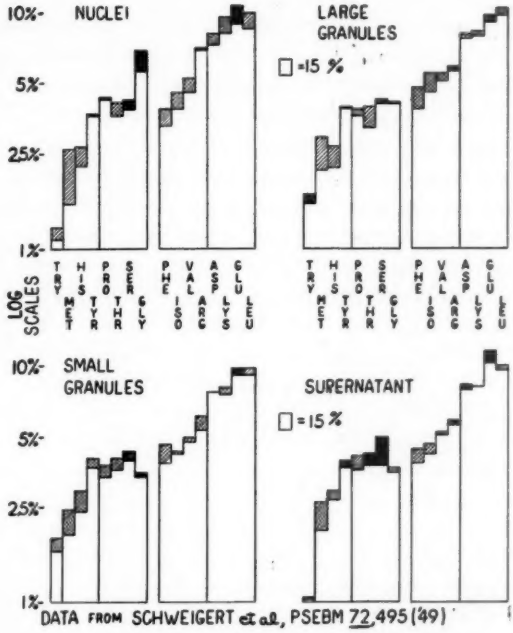

FIG. 3. Amino acid composition of morphological fractions of normal rat livers and rat hepatomas. Graphs constructed from data of Schweigert et al. (1949). Only the data on the liver tumors and on the livers of rats fed basal diet were used. The cancer values are superimposed on those of the corresponding normal tissue fraction. A higher value for the cancer tissue is shown by a black area, a lower value by a shaded area. Equal areas indicate equal percentage differences.

from one type of tissue fraction to another are more pronounced than the difference between the normal and its cancerous counterpart.

These and other recent studies have developed rather new perspectives regarding the composition of tissue proteins. The immunochemist is able to demonstrate high degrees of differentiation among tissue proteins of different species, different organs and even different individuals. But the analytical chemist, when he takes these proteins apart and sorts and counts the building stones, finds the same building blocks and finds

that nature uses them in very similar ratios for many different purposes. While the picture of the immuno-protein chemist is dominated by dissimilarities the picture of the analytical protein chemist is dominated by similarities.

To be sure we do find significant gross differences among non-cellular proteins, such as those of hair and other epidermal appendages, or those of connective tissue, or the storage proteins of seeds and eggs, or the secreted proteins like insulin and other hormones, or the circulating plasma proteins. But in order to qualify as a cellular protein, it would seem that certain over-all specifications of pattern have to be met and that the cellular proteins of cancer have to submit to this building code; or, perhaps, they insure their own sinister existence by complying with the over-all rules of construction. We might call this the amino acid distribution code of cellular protein construction.

It would then be because of this code that the protein differences which are indicated by the immunological test of antigenicity and which are suggested by the histological test of cellular architecture (i.e., cancer diagnosis), elude ordinary chemical analysis.

A point of view such as this prompted us several years ago to consider a new approach which would have to satisfy several requirements. We would have to compare a tissue of the highest possible degree of cellular homogeneity, with a homogeneous malignant tissue developing therefrom. Because of the amino acid building code we would have to concentrate on the study of intact proteins rather than their building blocks. Such studies are possible in principle through the physical methods of electrophoresis and ultracentrifugation which measure size, shape and electrical charge distribution of individual protein species and permit their separation according to these properties.

The more common sites of cancer are glandular tissues, such as liver or breast or lymphatics, or tissues of complicated architecture such as lungs or epidermis. To avoid the complexities of these tissue types we considered striated muscle, as presumably the most homogeneous tissue of the mammalian

organism. Those who have investigated rates of protein metabolism have found muscle, which makes up more than one-half of the body substance, to be among the more inert tissues (Shemin and Rittenberg, 1944). Cancer of the muscle is extremely rare, and perhaps this is so because of its slow protein metabolism. However, even the muscle cell will yield to the insult of the carcinogenic hydrocarbon. On the basis of advice received from Dr. Lionel Strong, Dr. Briggs and Dr. Green at our Philadelphia Institute were able to induce with methylcholanthrene a rhabdomyosarcoma in the mouse, which now has been propagated with clockwork regularity by transplantation for many generations. And the problem of the control tissue, normal muscle, presents no difficulty.

When we planned this program, we were further attracted by the fact that more was already known about muscle proteins than about any other cellular proteins. We were not aware at that time (this was before the end of the war) of the brilliant advances made by Szent-György (1947) in this field. When these came to light they were obviously a very pleasant surprise and they have been of great help in the subsequent work. The work itself is entirely in the hands of Dr. Gail Miller (1950, a, b) and a detailed account of his initial findings is contained in his publications. I am indebted to Dr. Miller for providing me with a few slides which will illustrate what at the moment seems the most interesting aspect of this work. The tissues, that is, normal mouse muscle and rhabdomyosarcoma of the mouse, are pulverized in the frozen state and extracted with half-molar salt solutions. Electrophoresis of these extracts shows complex and different patterns. When the salt content of the extract of normal muscle is lowered by dialysis to 0.02 molar, myosin, and apparently only myosin, precipitates. When the cancer tissue extract is similarly treated a similar precipitate appears. These myosin precipitates were then purified and separately subjected to electrophoresis. Their mobilities differed by about 20 per cent.

Figure 4 shows the electrophoretic pattern of the normal myosin. Figure 5 shows the pattern of the tumor myosin. Because of certain experimental variables it was necessary to

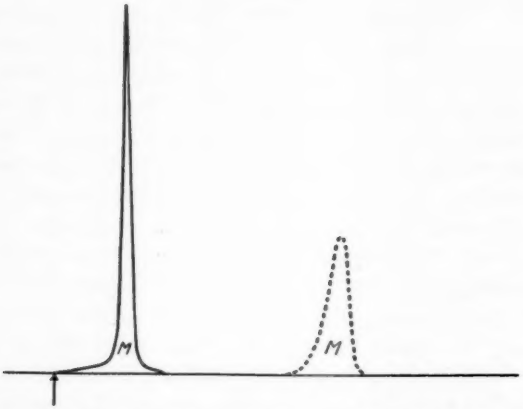

FIG. 4. Electrophoretic pattern of myosin from normal mouse muscle, according to Miller et al. (1950, b).

FIG. 5. Electrophoretic pattern of myosin from mouse rhabdomyosarcoma, according to Miller et al. (1950, b).

run mixtures of the two fractions in order to settle with certainty the question of their actual identity or non-identity. Figure 6 shows the result. There seems to be no question that

FIG. 6. Electrophoretic pattern of a mixture of the two myosin preparations shown in Figures 5 and 6.

that the two fractions differ in their electrophoretic properties. Both appear to be homogeneous fractions, and both have the characteristic solubility properties of a myosin. As to the other

protein components of these tissues work on their resolution is proceeding. At the moment it can be said that of some seven components which electrophoresis reveals in the tumor extracts and some four components of the normal extract no two appear to be identical. Analogous components are certainly not ruled out pending further work. However, the picture revealed in these studies by the electrophoretic approach appears to be dominated by dissimilarities between the cancer tissue and the homologous normal tissue, rather than by the similarities which characterize the results of chemical analysis so far.

REFERENCES

Bullough, W. S., and E. A. Eisa: The Effects of a Graded Series of Restricted Diets on Epidermal Mitotic Activity in the Mouse. *Brit. J. Cancer*, 4:321–336, 1950.

Dublin, L. I.: Longevity in Retrospect and in Prospect. In: Problems of Ageing. *Biol. and Med. Aspects.* Second Edition. Ed. by E. V. Cowdry, Baltimore. Williams and Wilkins Co., pp. 91–110, 1942.

Dunning, W. F., and M. R. Curtis: The Respective Roles of Longevity and Genetic Specificity in the Occurrence of Spontaneous Tumors in the Hybrids Between Two Inbred Lines of Rats. *Cancer Research*, 6:61–81, 1946.

Earle, W. R.: Production of Malignancy in Vitro. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells. *J. Nat. Canc. Inst.*, 4:165–212. 1943–44.

Gey, G. O.: Cytological and Cultural Observations on Transplantable Rat Sarcomata Produced by the Inoculation of Altered Normal Cells Maintained in Continuous Culture. *Proc. 34th Meeting Am. Assoc. Canc. Res., Cancer Research*, 1:737, 1941.

Greenstein, J. P.: Recent Progress in Tumor Enzymology. *Advances in Enzymology*. Ed. by F. F. Nord and C. H. Werkman, 3:315–348, 1943.

Lindsey, D., and E. M. Cohart: Incidence of Cancer in American Males. 15,000,000 Man-Years of Aggregate Experience, United States Army, 1944–1945. *Cancer*, 3:945–959, 1950.

McCay, C. M.: Chemical Aspects of Ageing and the Effect of Diet Upon Ageing. In: Problems of Ageing. *Biol. and Med. Aspects.* Second Edition. Ed. by E. V. Cowdry, Baltimore. Williams and Wilkins Co., pp. 680–727, 1942.

Miller, G. L., E. U. Green, J. J. Kolb, and E. E. Miller: Studies on the Proteins of Rhabdomyosarcoma and Normal Muscle of Mice. I. Gross Composition, Extractions with 0.5 *M* KCl, and Fractionations by Differential Centrifugation and Dialysis. *Cancer Research*, 10:141–147, 1950 (a).

Miller, G. L., E. U. Green, E. E. Miller, and J. J. Kolb: Studies on the Proteins of Rhabdomyosarcoma and Normal Muscle of Mice. II. Electrophoretic and Viscosimetric Measurements. *Cancer Research*, 10:148–154, 1950 (b).

Pollack, M. A., A. Taylor, and R. J. Williams: B Vitamins in Human, Rat and Mouse Neoplasms. *The University of Texas Publications*, 4237:56–71, 1942.

Roberts, E., and S. Frankel: Free Amino Acids in Normal and Neoplastic Tissues of Mice as Studied by Paper Chromatography. *Cancer Research*, 9:645–648, 1949.

Sauberlich, H. E., and C. A. Baumann: The Amino Acid Content of Certain Normal and Neoplastic Tissues. *Cancer Research*, 11:67–71, 1951.

Schweigert, B. S., B. T. Guthneck, J. M. Price, J. A. Miller, and E. C. Miller: Amino Acid Composition of Morphological Fractions of Rat Livers and Induced Liver Tumors. *Proc. Soc. Exp. Biol. Med.*, 72:495–501, 1949.

Shemin, D., and D. Rittenberg: Some Interrelationships in General Nitrogen Metabolism. *J. Biol. Chem.*, 153:401–421, 1944.

Szent-Györgyi, A.: *Chemistry of Muscular Contraction.* New York: Academic Press, Inc., 1947.

Tannenbaum, A.: Relationship of Body Weight to Cancer Incidence. *Arch. Path.*, 30:509–517, 1940.

Tannenbaum, A.: The Genesis and Growth of Tumors. II. Effects of Caloric Restriction per se. *Cancer Research*, 2:460–467, 1942.

Toennies, G., J. J. Kolb, and D. L. Gallant: Unpublished data.

Wenner, C. E., M. A. Spirtes, and S. Weinhouse: Enzymes of the Citric Acid Cycle in Tumors. *J. Am. Chem. Soc.*, 72: 4333, 1950.

Weinhouse, S., R. H. Millington, and C. E. Wenner: Occurrence of the Citric Acid Cycle in Tumors. *J. Am. Chem. Soc.*, 72:4332–3, 1950.

IN VITRO PROTEIN SYNTHESIS AND TURNOVER IN MOUSE TISSUES*

RICHARD J. WINZLER

The problem of cancer is essentially that of uncontrolled tissue growth and the synthesis of the constituents of the malignant cells. Since proteins are among the most important of these constituents, studies which are directed toward elucidation of the mechanisms of protein systhesis are of fundamental importance in cancer research.

Our laboratories (Pearson, 1950) have for the past several years been conducting a program involving the study of the *in vitro* effects of a neurotropic virus (Theiler's GD VII mouse encephalomyelitis virus) on the metabolism of minced, one-day-old mouse brain, a tissue in which this virus is readily propagated (Pearson, 1950). The experimental procedure involves the sterile removal of the brain from newborn mice. An entire brain (50 to 70 mg. of tissue) is minced and suspended in 3 ml. of a simple medium containing only salts and glucose (Simms solution). Such minces are inoculated with the virus and incubated at 35° C., usually for 24 hours. In this time the virus titer is increased by 1,000 to 10,000 fold as measured by intracerebral inoculation of the supernatant solution into mice. It was found that infection with virus elicited no detectable difference in the extent of glucose utilization, of lactic acid production, or of oxygen consumption in the control (uninfected) and the infected tissues during the period of maximal virus growth (Pearson and Winzler, 1949). The first table shows the summary of a series of determinations of this sort, the oxygen consumption being measured manometrically in

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Warburg vessels and glucose disappearance and lactate production being chemically determined. In spite of this failure to demonstrate quantitative alterations in the energy yielding

TABLE I

Effect of Theiler's GD VII Virus on Respiration and Glycolysis of Mouse Brain

	Total 0_2 Consumed uL/100 mg.	Total Gluc. used mg./100 mg.	Total Lactate produced mg./100 mg.	Virus Titer
Control	910	1.98	2.04	.....
Infected	935	1.94	2.12	100,000

24 hour incubation in Warburg flasks. Virus titer is the dilution of the supernatant which killed half of a group of mice injected intracerebrally with 0.03 ml. Initial titer 100.

reaction of the host tissue by virus infection, it was clear from the increase in virus titer that a new and foreign protein was being produced in the infected tissues. Consequently it was felt desirable to investigate the turnover of some of the cellular components, since such studies give a more satisfactory indication of the synthetic reactions proceeding within a tissue than does respiration and glycolysis.

Experiments of this sort were first carried out using radioactive phosphate, tracer amounts of this compound being added to the incubation medium. After incubation in the presence or absence of virus the tissues were fractionated into the inorganic-, organic acid-soluble-, phospholipid-, total protein-bound-, pentose nucleic acid-, desoxypentose nucleic acid-, and phosphoprotein phosphorus fractions by slight modifications of established methods. The specific activity of each fraction was determined and related to the inorganic phosphate as 100 (Relative Specific Activity). Table II shows data (Rafelson, *et al.*, 1949) which indicate that the infection of the host tissue with this virus markedly stimulates the incorporation of radioactive phosphate into the phospholipid and the total protein-bound fractions, the latter effect being almost entirely limited to stimulation of the turnover of the pentose nucleic acid fraction.

These results raised the question of whether the increased phosphorus turnover of the lipid and protein fractions in the

TABLE II

Effect of Theiler's GD VII Virus on $P^{32}O_4$ Metabolism of Mouse Brain

Fraction	*Control R.S.A.	*Control ugmP/100 mg.	*Infected R.S.A.	*Infected ugmP/100 mg.
Inorganic	100.		100.	
Org. acid sol...	53.5 ±2.6	31.0±1.0	51.7 ±1.4	25.0±1.1
Org. acid sol....	53.5 ±2.6†	31.0±1.0†	51.7 ±1.4†	25.0±1.1†
Phospholipid ..	2.91±0.04	50.0±1.1	4.15±0.05	48.0±1.5
Tot. prot. bnd..	2.32±0.06	40.2±0.9	3.55±0.17	42.8±0.3
P.N.A.	2.38±0.04	15.5±0.5	3.99±0.07	21.4±1.2
D.N.A.	1.16±0.04	13.0±0.3	1.18±0.04	9.6±0.5
Phosphoprotein	0.84±0.03	5.5±0.5	0.89±0.02	4.5±0.5
P.I.P.	4.57±0.25	6.8±0.6	4.94±0.4	7.1±0.6

24 hour incubation.

$$*\text{R. S. A.} = \frac{\text{cts/ugm P in fraction}}{\text{cts/ugm inorganic P}}$$

$$\dagger\text{S.E.M.} = \sqrt{(\Sigma d^2/(n\text{-}1))/n}$$

virus-infected cultures was associated with phosphate components alone, or whether the turnover of the carbon skeleton of these fractions was also stimulated. The incubations were therefore carried out in media containing glucose uniformly-labeled with radioactive carbon. Radioactive carbon from this labeled glucose was observed to be incorporated into the lipid and the protein fractions of the control (uninfected) tissues. *In vitro* infection with Theiler's GD VII virus significantly increased the extent of incorporation of radioactive carbon into the proteins of the host tissue but decreased the incorporation into the lipids (Rafelson, *et al.*, 1949). The effect is not due to the fixation of carbon dioxide (produced from oxidation of radioglucose) since much less radiocarbon appears in the proteins of tissue incubated with radiocarbonate, and the virus has no influence on this process. Some data summarizing this work are shown in Table III.

The appearance of radioactive carbon in the proteins and the stimulating effect of virus propagation raised the question of which constituents of the protein fraction had incorporated glucose fragments and also the question of whether the virus infection had influenced the turnover of specific constituents. It therefore became desirable to separate the amino acids from the cultures incubated with radioglucose, and to determine

TABLE III

Effect of Theiler's GD VII Virus on Metabolism of Radioglucose or Radiobicarbonate by Mouse Brain

	Glucose* (1,200,000 cts.)		$NaHCO_3$ (3,000,000 cts.)	
	Control	Infected	Control	Infected
CO_2	1.87±0.1[1]	1.80±0.1	93.9 ±0.85	94.8 ±1.5
Acid sol. ..	91.15±0.2	91.23±0.2	0.49±0.05	0.55±0.05
Protein ..	0.71±0.1	1.57±0.1	0.16±0.02	0.19±0.02
Lipid	1.28±0.1	0.59±0.1	0.34±0.03	0.30±0.03
Recovery..	95.01	95.19	94.89	95.84

24 hour incubations. Values expressed as percent of added counts recovered.

[1] SEM $= \sqrt{(\Sigma d^2/n\text{-}1)/n}$

their radioactivity. The elegant method of Moore and Stein (1948) using starch columns for the separation of amino acids seemed admirably suited to this purpose. Acid hydrolysates of the proteins from one-day-old mouse brain tissue incubated with radioactive glucose in the presence and absence of Theiler's GD VII virus were prepared and chromatographed by the methods of Moore and Stein. The amino acid content and the radioactivity were determined on each effluent fraction. The results of an entirely typical experiment are shown in Figure 1.

An unexpected observation shown in Figure 1 is that all but two of the amino acids effluent peaks were associated with a radioactive peak. The association of effluent radioactivity with an amino acid cannot be considered positive proof that the amino acid is itself radioactive. This question has been examined critically by running radioautographs after the partition chromatography of the effluent peaks and acting the identical of the R_f for radioactivity and the amino acids. Several amino acids (leucine, isoleucine, phenylalanine and methionine) were recrystallized to constant specific activity. Preliminary determinations of the location of the isotopic carbon have been made with the above recrystallized amino acids. About half of the total radioactivity is in the carboxyl carbons of leucine, isoleucine and phenylalamine whereas lysine contains radioactive carbon only in the carboxyl group. About twenty-five per cent of the methionine radioactivity is in the carboxyl carbon, and another twenty-five per cent is in the methyl group.

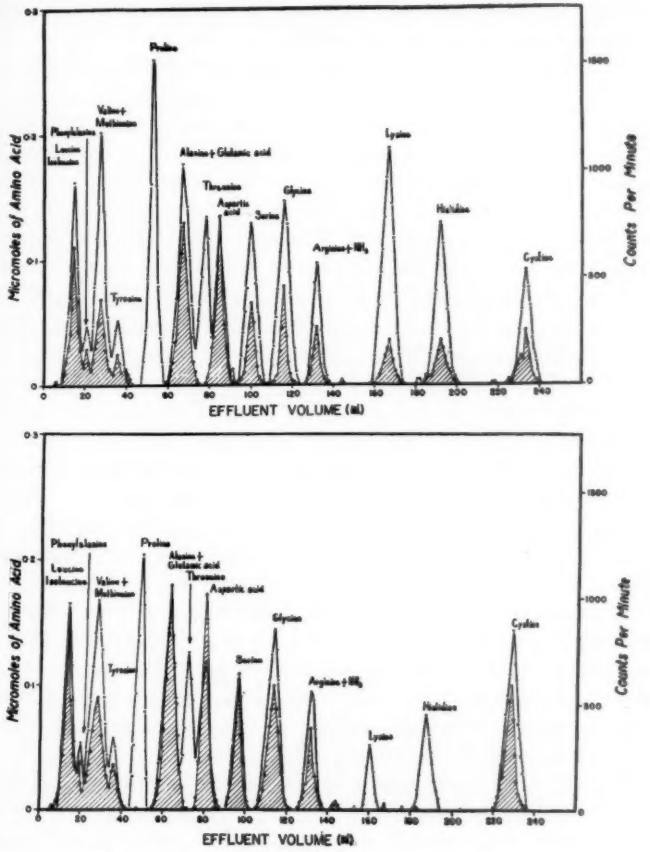

FIGURE 1

Figure 1, which is typical of all of the experiments that have been carried out shows that, with three exceptions, the amounts of all of the amino acids is not different in the control and virus infected tissues. These exceptions are lysine and histidine which are decreased, and cystine which is increased in the infected cultures. The cystine peak has been shown by paper chromatography to contain at least two components other than

cystine, and the apparent increase in cystine may possibly reflect changes in the contaminants.

From Figure 1, it is also evident that the specific activity of most of the amino acids is increased by virus infection, indicating more rapid turnover. However, lysine and histidine while they contain significant radioactivity in the control tissue, fail to take up any radiocarbon from glucose after infection with Theiler's virus. These data indicate that *in vitro* infection of mouse brain with Theiler's GD VII virus significantly alters the protein metabolism of the host tissue.

It has not been possible to assess the direct contribution of virus protein to the changes that have been demonstrated, since the virus has not yet been isolated. However, it appears most likely that the virus effect is mainly through an alteration of the protein metabolism of the host tissue. This is in part based on the magnitude of the changes that have been found.

The specific activity of the original starting glucose used in these experiments was 436,000 counts/min./mg. or 13,050 counts/min./micromole of carbon. The specific activity of aspartic acid in the control experiment of Figure 1 was 4,560 counts/min./micromole or 1,140 counts/min./micromole carbon. Assuming equal distribution of the radioactivity in the asparate, the results would indicate a replacement of 8.8% of the carbons of aspartic acid by carbons from radioglucose. Similar calculations can be made for certain of the other amino acids knowing the specific activity of the amino acid and something of the distribution of its radiocarbon.

The per cent replacement of some of the amino acids calculated similarly is shown in Table IV. In all cases from 5 to 10% of the amino acids are replaced by carbon from radioglucose. If this represented amino acid synthesis without concommitant breakdown, the change in amino acid would have been detectable in the assay method employed. Since no such increase has been found in the amounts of any of the amino acids (with the possible exception of cystine), it is concluded that no net synthesis of amino acids from glucose had occurred in any of the experiments.

The production of a foreign protein, however, had occurred in the infected series of flasks, since a substantial increase in

TABLE IV

Turnover of Some Amino Acids in Mouse Brain Incubated with Radioactive Glucose

Amino Acid	cts./uM AA	Assumptions	cts./um C	% Repl.
ASPARTATE	4560	Even distribution	1140	8.8
GLYCINE	2290	Even distribution	1145	8.8
THE LEUCINES	2870	50% in COOH	1435	11.0
LYSINE	780	100% in COOH	780	6.0
PHENYLALANINE	2620	50% in COOH	1310	10.0
SERINE	2210	Even distribution	733	5.6
TYROSINE	2110	Side chain only	703	5.4

Control 24 hour incubation with C^{14} glucose with a S.A. of 436,000 cts./min./mg. or 13,050 cts./min./uM carbon.

virus titer has invariably been noted. The amounts of this foreign protein released into the supernatant solution is exceedingly small. On the basis of available evidence it is not possible to determine whether the virus is formed by complete synthesis from amino acid and nucleic acid precursors or is formed by alteration of pre-existing proteins and nucleic acids. Clarification of this point will depend to a large extent upon the isolation of purified virus.

The observation that the essential amino acids in the *in vitro* system employed in these studies, were in some sort of metabolic equilibrium with glucose, it was interesting in view of the well established observation that these amino acids must be supplied in the diet for growth and maintenance. The appearance of carbon from radioactive glucose in the brain proteins of the intact one-day-old mouse has therefore been studied. New-born mice were injected intraperitoneally at two-hour intervals with radioactive glucose, and at the end of 24 hours the brain proteins were isolated, hydrolized, and chromatographed as in the *in vitro* experiments. In this preliminary work radioactive carbon has been found primarily in glutamic acid, aspartic acid, alanine, serine, glycine, and arginine. However, small but probably significant amounts were also found associated with the leucines, valine plus methionine, lysine, histidine, and cystine peaks. It has been clear that the *in vitro* conditions used in these experiments leads to a distinctly different distribution of radioactive carbon from that found in the intact mouse, and that considerable care

must be exercised in transferring results obtained *in vitro* too freely to *in vivo* systems.

These experiments have shown that protein metabolism of tissue may be distinctly altered by infection with a virus. The differences appear to involve both quantitative and qualitative changes, and result in the production of a protein not ordinarily observed in the tissues.

Sufficient information is not yet at hand to demonstrate unequivocally the presence of abnormal proteins in malignant tissue (Toennies, 1947; Winzler, in press). However, the suggestion that proteins may be altered during carcinogenesis has been advanced by Potter (1943), and has gained some experimental support in recent studies on aminoazo dye carcinogenesis. The possibility may be entertained that the transformation of normal to malignant tissue involves an altered protein metabolism of the sort demonstrated in the studies that have been described. This may possibly involve the appearance of an abnormal protein perhaps a sort of "cancer virus" (Miller, *et al.*, 1949), capable of redirecting the protein metabolism of the normal tissue into pathways characteristic of malignancy. This hypothesis though vulnerable, provides a working framework around which an experimental approach to the problem of protein metabolism in cancer can be built.

REFERENCES

Miller, E. E., J. A. Miller, R. W. Sapp, and G. M. Weber: *Cancer Research*, 9:336, 1949.

Moore, S., and W. H. Stein: *J. Biol. Chem.*, 176:337, 367, 1948.

Pearson, H. J., and R. J. Winzler: *J. Biol. Chem.*, 181:577, 1949.

Pearson, H. J.: *J. Immunol.*, 64:447, 1950.

Potter, V. R.: *Cancer Research*, 3:358, 1943.

Potter, V. R.: *Advances in Enzymol.*, 4:201, 1944.

Rafelson, M. E., R. J. Winzler, and H. E. Pearson: *J. Biol. Chem.*, 181: 583, 1949.

Rafelson, M. E., R. J. Winzler, and H. E. Pearson: *J. Biol. Chem.*, 181:595, 1945.

Stein, W. H., and S. Moore: *J. Biol. Chem.*, 178:79, 1949.

Toennies, G.: *Cancer Research*, 7:193, 1947.

Winzler, R. J.: *Physiology of Cancer*, In Press.

STUDIES ON *IN VITRO* METABOLISM OF THE RAT HEPATOMA*

PAUL C. ZAMECNIK

It has been stated that the essential starting point in any problem is to ask the right question. An important dual question in the cancer problem is why malignant cells continue to grow at an excessive rate, and why they invade normal tissue.

In searching for an answer, it is convenient to study the process of carcinogenesis in a neoplasm easily produced in a common laboratory animal. For this purpose we have chosen the primary *p*-dimethylaminoazobenzene-induced hepatoma in the rat. If weanling female rats of the Harvard Colony† are fed *ad libitum* on a diet low in protein and riboflavin, which contains 0.06 per cent of this dye, approximately 90 per cent of the animals develop malignant hepatic neoplasms by the end of a six-month feeding period. If the animals are returned to a normal diet by the end of the fourth month, a smaller number of isolated, well-circumscribed tumors appear by the sixth month. Histologically, these are similar to the hepatic tumors described by Edwards and White (1941) as hepatoma type 2, consisting in large part of sheets of neoplastic cells resembling parenchymal liver cells, interspersed with smaller areas of adenocarcinoma and bile duct carcinoma. The tumor nodules are pearly white, and free from gross evidence of necrosis until their diameter exceeds 1 centimeter. We have therefore endeavored to use the smaller tumors, have avoided necrotic tissue, and have frequently checked the histology of the tissue adjacent to that used in biochemical studies.

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†These rats were obtained originally from Sprague-Dawley and have been inbred periodically in Hisaw's laboratory by brother and sister matings during the last 15 years.

A quarter of a century ago, Otto Warburg (1931) contributed importantly to cancer research in pointing out the heightened capacity of slices of tumor tissues, in comparison with slices of most normal tissues, to degrade glucose to lactic acid in the presence of air. This increased aerobic glycolytic ability of tumors was the subject of much further investigation during the next fifteen years, but little other than certain qualifying phrases has been added to Warburg's early observations.

It has recently become possible, however, to reopen the quest for an explanation of the increased ability of the neoplasm to ferment glucose in the presence of air. By means of a biosynthetic process, $C^{14}O_2$ may be built into C^{14}-labeled starch by bean leaves (Putnam, *et al.*, 1948; Villee and Hastings, 1949). The starch is purified, hydrolyzed to glucose, and separated from traces of labeled contaminants by paper chromatography (Partridge, 1949; Zamecnik, *et al.*, 1951). C^{14} glucose prepared in this way has been found to be randomly labeled, in all six carbon positions, a situation advantageous for experiments designed to trace the fate of glucose fragments resulting from degradation of glucose by tissue slices.

In our laboratory, as well as in others in the country, slices of normal and malignant tissues have been incubated in C^{14}-glucose, and the extent of conversion of the Cvd *to protein* (Winzler, *et al.*, 1950; Zamecnik and Stephenson, 1950) lipid, lactate, and CO_2 (Weinhouse, *et al.*, 1950; Olson, *et al.*, 1951) has been measured, over a 1–2 hour period. Experiments of this type confirm Warburg's old findings of increased lactic acid production in tumors, and contribute the additional information in the case of this particular neoplasm that the carbon skeleton of glucose is converted to protein, lipid, and CO_2 to a greater extent than occurs in the control liver slice.

Warburg found that certain normal tissues such as brain and renal medulla shared with neoplastic tissues a high aerobic glycolytic rate. In slice experiments in which the extent of conversion of C^{14}-glucose to protein over a 2 hour period was compared in brain, kidney, medulla, hepatoma, and two types of control hepatic tissue, the greatest conversion of the glucose skeleton to protein was found in the hepatoma slice.

To provide an extension of this work, Dr. R. B. Loftfield synthesized carbonyl-labeled C^{14}-pyruvate, and carboxl-labeled C^{14}-lactate. Slice experiments were then carried out, using normal and neoplastic tissues (Zamecnik, *et al.*, 1951). In comparison with the normal hepatic slice, the malignant one degrades glucose to pyruvate more rapidly, converts pyruvate to glucose less rapidly, converts pyruvate to protein more rapidly, and degrades pyruvate to CO_2 a little more rapidly. The pyruvate $\leftrightharpoons$ lactate equilibrium itself is not appreciably disturbed, if one uses either one of those compounds as a labeled indicator.

These slice experiments focus attention on the problem of protein construction in the malignant cell, suggesting that a disproportionate amount of the carbon skeleton of glucose is converted to structural proteins.

We have endeavored to study protein synthesis in malignant tissue, and indeed this was the point to which our carbon 14 studies were originally directed. It was clear to us, however (Zamecnik and Frantz, 1949) that the process of incorporation of a labeled amino acid into protein might not be the same as protein synthesis. For example, incorporation might be achieved in part by an exchange process, involving no net synthesis of new peptide bonds. We have, however, continued to consider the incorporation of a labeled amino acid into protein under the conditions of our experiments as one parameter of protein synthesis.

We have found that if slices of normal and malignant hepatic tissue are incubated in a simple Krebs-Ringer-phosphate medium in the presence of oxygen, there occurs a greater rate of incorporation of labeled amino acid into the protein of the malignant tissue slice than into the protein of the normal control liver slice. The rate of incorporation of C^{14}-alanine into the protein of fetal rat liver is, however, in the same general range as that of the hepatoma.

The same type of result has been obtained in slice experiments in which C^{14}-leucine, C^{14}-glycine, or a fairly complete mixture of C^{14}-labeled amino acids (obtained by biosynthesis) (Frantz and Feigelman, 1950) were used. Several other neoplasms have likewise been studied, and found to incorporate

the labeled amino acids into protein at a more rapid rate than the non-malignant tissues of origin of the tumors in the same animal. Specifically, ten carcinomas of the breast in humans and two fibrosarcomas have been studied in this way in collaboration with Dr. Ira T. Nathanson (unpublished data). Normal lactating mammary tissue slices, however, incorporated C^{14}-alanine into protein as rapidly as did the adenocarcinoma tissue from two patients. Here it is likely that casein, a secretory protein, was being synthesized at a rapid rate by the lactating normal tissue, whereas non-secretory structural proteins were being synthesized by the carcinoma tissue. This, however, is speculation.

It became clear to us some time ago that further progress in understanding the deranged protein metabolism of neoplastic tissue would probably depend on uncovering more knowledge of the mechanism of protein synthesis as carried out within the normal cell. Much effort has been directed toward isolation of a system capable of synthesizing protein, or at least of incorporating labeled amino acids into protein, in the absence of living cells. Drs. Greenberg's (1948) and Borsook's (1949) laboratories have described the incorporation of labeled amino acids into proteins in cell free homogenates of tissues.

A mammalian liver cell consists of several distinct particulate components—the cell membrane, the nucleus, the mitochondria, and the microsomes. Dr. Elizabeth B. Keller (1951) of our laboratories injected C^{14}-leucine intravenously into a series of rats, and then separated out several fractions from the livers by homogenization of the cells, followed by differential centrifugation. She found that the fastest rate of incorporation of C^{14}-leucine into protein occurred in the microsome fraction. This finding agrees with that of Borsook (1950), and is of particular interest inasmuch as microsomes are rich in ribosenucleic acid, and nucleic acids have been shown by optical methods (Caspersson, 1950) to be present in great concentration at sites where protein synthesis is presumed to occur.

Dr. Philip Siekevitz (1951) of our laboratories has carried this type of experiment a step further. He has homogenized liver cells in order to break up the cell membranes and destroy much of the cell architecture, and then has incubated the

homogenate with C^{14}-alanine. In this cell-free system, incorporation of the labeled amino acid into protein has been found, and upon fractionation of the particles, the greatest rate of incorporation has again been found to occur in the microsome fraction. Dr. Siekevitz was then encouraged to try incubation of separate particulate components of the liver with C^{14}-alanine. A negligible incorporation occurred when either mitochondria, microsomes, or the supernatant non-sedimentable fraction of the liver was incubated alone. When the mitochondrial fraction was added to the microsome-rich fraction, and these two were incubated together (in the presence of oxygen, inorganic phosphate, and a carbohydrate substrate), however, incorporation of the labeled alanine into protein occurred.

These interesting experiments suggest that by oxidative processes the mitochondria generate energy which is used to incorporate amino acid into protein. The actual incorporation, however, appears to take place in the microsomes. If the mitochondrial function is interferred with—by addition of dinitrophenol, or previous heat inactivation of the mitochondria—the incorporation does not occur. It must be emphasized that the nature of the incorporation process is not understood, and one is not justified in calling it protein synthesis. Nevertheless, this work appears to provide an opening wedge for further studies on peptide bond synthesis in cell-free systems.

To summarize our findings on the metabolism of tumor tissue, it may be stated that no new pathways have been uncovered by which neoplastic tissues derive energy from foodstuffs, or convert simple building blocks into complex cell structures. The experiments do suggest that an undue amount of the energy available to the malignant cell from glycolytic and oxidative processes is funneled into the synthesis of proteins essential for cell division and growth.

REFERENCES

Borsook, H., C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy: *J. Biol. Chem.*, 179, 689, 1949.

Borsook, H., C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy: *J. Biol. Chem.*, 187, 839, 1950.

Caspersson, T. O.: *Cell Growth and Cell Function.* W. W. Norton & Co., New York, 1950.
Edwards, J. E., and J. White: *J. Nat. Cancer Inst.*, 2:157, 1941.
Frantz, I. D., Jr., and H. Feigelman: *Cancer Res.*, 9:619, 1950.
Greenberg, D. M., F. Friedberg, M. P. Schulman, and T. Winnick: Cold Spring Harbor Sympos. *Quant. Biol.*, 13, 113, 1948.
Keller, E. B.: *Fed. Proc.*, 10, 206, 1951.
Olson, R. E., E. G. Hirsch, and M. C. Jones: *Fed. Proc.*, 10, 230, 1951.
Partridge, S. M.: *Nature*, 164, 4167, 1949.
Putnam, E. W., W. Z. Hassid, G. Krotkov, and H. A. Barker: *J. Biol. Chem.*, 173, 785, 1948.
Siekevitz, P., and P. C. Zamecnik: *Fed. Proc.*, 10, 246, 1951.
Villee, C. A., and A. B. Hastings: *J. Biol. Chem.*, 179, 673, 1949.
Warburg, O.: *The Metabolism of Tumours*, translated by F. Dickens. R. R. Smith, Inc., New York, 1931.
Weinhouse, S., R. H. Millington, and C. E. Wenner: *J. Am. Chem. Soc.*, 72, 4332, 1950.
Winzler, R. W., R. Krause, and G. G. Slater: *Cancer Res.*, 10, 249, 1950.
Zamecnik, P. C., and I. D. Frantz, Jr.: Cold Spring Harbor Sympos. *Quant. Biol.*, 14, 199, 1949.
Zamecnik, P. C., and M. L. Stephenson: *Cancer Res.*, 10, 251, 1950.
Zamecnik, P. C., R. B. Loftfield, M. L. Stephenson, and J. M. Steele: *Cancer Res.*, 11, 592, 1951.